

METHODS FOR USING CHEMOKINE TECK

This application is a divisional application of U.S.S.N. 10/039,659, filed January 3, 2002, now allowed
5 which is a divisional application of U.S.S.N. 08/887,977 filed July 3, 1997, now abandoned which claims the benefit of U.S.S.N. 60/021,664 filed July 5, 1996; U.S.S.N. 60/028,329 filed October 11, 1996; and U.S.S.N. 60/048,593 filed June 4, 1997.

10

All references cited herein are incorporated in their entirety by reference.

FIELD OF THE INVENTION

15 The present invention relates to compositions related to proteins which function in controlling physiology, development, and/or differentiation of mammalian cells, e.g., cells of a mammalian immune system. In particular, it provides proteins and
20 mimetics which regulate physiology, development, differentiation, and function of various cell types, including hematopoietic cells. It also provides receptor reagents for chemokine-like proteins.

25

BACKGROUND OF THE INVENTION

The circulating component of the mammalian circulatory system comprises various cell types, including red and white blood cells of the erythroid or the myeloid cell lineages. See, e.g., Rapaport (1987)
30 Introduction to Hematology (2d ed.) Lippincott, Philadelphia, PA; Jandl (1987) Blood: Textbook of Hematology, Little, Brown and Co., Boston, MA.; and Paul (ed.) (1993) Fundamental Immunology 3d ed, Raven Press, N.Y. Progression through various stages of
35 differentiation are regulated by various signals provided to the cells, often mediated through a class of proteins known as the cytokines. Within this group of molecules as a further group known as the chemoattractant cytokines, or chemokines. See, e.g.,

Schall (1994) "The Chemokines" in Thomson (ed.) The Cytokine Handbook (2d ed.) Academic Press; and Schall and Bacon (1994) Current Opinion in Immunology 6:865-873.

5 Although the full spectrum of biological activities of the chemokines has not been extensively investigated, chemoattractant effects are recognized. The best known biological functions of these molecules relate to chemoattraction of leukocytes. However, new chemokines
10 are being discovered, and their biological effects on the various cells responsible for immunological responses are topics of continued study.

 Certain G-protein coupled receptors have also been characterized, presumably chemokine receptors. See,
15 e.g., Samson, et al. (1996) Biochemistry 35:3362-3367; and Rapport, et al. (1996) J. Leukocyte Biology 59:18-23.

 These observations indicate that other factors exist whose functions in hematopoiesis, immune
20 development, and leukocyte trafficking were heretofore unrecognized. These factors provide for biological activities whose spectra of effects are distinct from known differentiation, activation, or other signaling factors. The absence of knowledge about the structural,
25 biological, and physiological properties of the regulatory factors which regulate hematopoietic cell physiology in vivo prevents the modification of the effects of such factors. Thus, medical conditions where regulation of the development or physiology of relevant
30 cells is required remains unmanageable.

SUMMARY OF THE INVENTION

The present invention is based, in part, upon the discovery of new genes encoding chemokines, and new genes encoding various receptors for chemokines. It
5 embraces agonists and antagonists of the chemokines. In particular, sequences of various chemokines, e.g., designated Thymus Expressed ChemoKine (TECK); MIP-3 α ; MIP-3 β ; and 7 transmembrane receptors, designated "dendritic cell receptor for chemokine" (DC CR) and
10 "monocyte/dendritic cell receptor for chemokine" (M/DC CR); and mutations (muteins) of the respective natural sequences, fusion proteins, chemical mimetics, antibodies, and other structural or functional analogs are provided. It is also directed to isolated genes
15 encoding respective proteins of the invention. Various uses of these different protein or nucleic acid compositions are also provided.

The present invention provides a substantially pure or isolated polypeptide comprising a segment exhibiting
20 sequence homology to a corresponding portion of a mature TECK, MIP-3 α , MIP-3 β , DC CR, or M/DC CR, wherein the homology is at least about 70% identity and the portion is at least about 25 amino acids. Preferably, the protein further comprises a second segment exhibiting at
25 least about 90% identity over at least 9 amino acids; or at least about 80% identity over at least 17 amino acids. In other preferred embodiments, the polypeptide: is from a warm blooded animal selected from the group of birds and mammals, including a mouse or human; comprises
30 a natural sequence from Tables 1 through 5; exhibits a post-translational modification pattern distinct from a natural form of the polypeptide; is made by expression of a recombinant nucleic acid; comprises synthetic sequence; is detectably labeled; is conjugated to a
35 solid substrate; is conjugated to another chemical moiety; is a fusion protein; is in a denatured conformation, including detergent denaturation; further comprises an epitope tag; is an immunogenic polypeptide; has a defined homogeneous molecular weight; is useful as

a carbon source; is an allelic variant of SEQ ID NO: 2, 4, 6, 8, 10, or 12; is a 3-fold or less substituted form of a natural sequence; is in a sterile composition; is in a buffered solution or suspension; is in a regulated
5 release device; comprises a post-translational modification; is in a cell; or is in a kit which further comprises instructions for use or disposal of reagents therein.

In other aspects, the invention provides an
10 isolated or recombinant nucleic acid encoding such protein, where the portion consists of sequence from the coding region of SEQ ID NO: 1, 3, 5, 7, 9, or 11. Other aspects include such nucleic acids which: exhibit at least about 80% identity to a natural cDNA encoding said
15 segment; is in an expression vector; further comprises a promoter; further comprises an origin of replication; is from a natural source; is detectably labeled; comprises synthetic nucleotide sequence; is less than 6 kb; is from a mammal; comprises a natural full length mature
20 coding sequence; is in a kit, which also comprises instructions for use or disposal of reagents therein; is a specific hybridization probe for a gene encoding the protein; is a PCR product; or is in a cell. The invention also provides a method of using a purified
25 nucleic acid by expressing the nucleic acid to produce a protein.

Alternatively, the invention provides an isolated or recombinant nucleic acid which encodes at least eight consecutive residues of SEQ ID NO: 2, 4, 6, 8, 10, or
30 12. Preferably, that nucleic acid encodes at least: twelve consecutive residues from SEQ ID NO: 2, and further comprises a coding sequence of at least 17 nucleotides from SEQ ID NO: 1; twelve consecutive residues from SEQ ID NO: 4, and further comprises a
35 coding sequence of at least 17 nucleotides from SEQ ID NO: 3; twelve consecutive residues from SEQ ID NO: 6, and further comprises a coding sequence of at least 17 nucleotides from SEQ ID NO: 5; twelve consecutive residues from SEQ ID NO: 8, and further comprises a

coding sequence of at least 17 nucleotides from SEQ ID NO: 7; twelve consecutive residues from SEQ ID NO: 10, and further comprises a coding sequence of at least 17 nucleotides from SEQ ID NO: 9; or twelve consecutive residues from SEQ ID NO: 12, and further comprises a coding sequence of at least 17 nucleotides from SEQ ID NO: 11. In other preferred embodiments, the nucleic acid: exhibits at least about 80% identity to a natural cDNA encoding the segment; is in an expression vector; further comprises a promoter; further comprises an origin of replication; encodes a 3-fold or less substituted sequence from a natural sequence; is from a natural source; is detectably labeled; comprises synthetic nucleotide sequence; is less than 6 kb; is from a mammal; is attached to a solid substrate, including in a Southern or Northern blot; comprises a natural full length coding sequence; is in a cell; or is in a detection kit, which also comprises instructions for use or disposal of reagents therein. Further embodiments include a nucleic acid which hybridizes under stringent wash conditions of 55° C and less than 150 mM salt to the nucleic acid; while preferred embodiments include those which exhibit at least about 85% identity over a stretch of at least about 30 nucleotides to a primate sequence of SEQ ID NO: 1, 3, 5, 7, 9, or 11; or where the identity is at least 90%; or the stretch is at least 75 nucleotides; or where the identity is at least 95%; or the stretch is at least 100 nucleotides.

In other embodiments, the invention provides a binding compound comprising an antigen binding fragment from an antibody which binds to a mature TECK, MIP-3 α , MIP-3 β , DC CR, or M/DC CR protein. In various embodiments, the binding compound is one wherein: the polypeptide is a mouse or human protein; the antibody is raised against a mature peptide sequence of Tables 1 through 5; the antibody is a monoclonal antibody; the binding compound is attached to a solid substrate; the binding compound is in a sterile composition; the

binding compound binds to a denatured antigen, including a detergent denatured antigen; the binding compound is detectably labeled; the binding compound is an Fv, Fab, or Fab2 fragment; the binding compound is conjugated to a chemical moiety; the binding compound is in a detection kit which also comprises instructions for use or disposal of reagents therein.

The invention also provides a cell which makes the antibody.

10 The invention embraces methods of purifying a polypeptide using a binding compound to specifically separate said polypeptides from others; of generating an antigen-binding compound complex comprising the step of contacting a sample comprising the antigen to a sample
15 comprising a binding compound; or of modulating physiology or development of a cell expressing a receptor for a chemokine selected from TECK, MIP-3 α , or MIP-3 β ; the method comprising contacting the cell with a composition comprising an agonist or mutein of said
20 chemokine or an antibody antagonist of the chemokine. In certain embodiments of the method, the cell is a macrophage, lymphocyte, or eosinophil; or the physiology is a cellular calcium flux, a chemoattractant response, cellular morphology modification responses,
25 phosphoinositide lipid turnover, or an antiviral response. In other embodiments, the receptor is DC CR and the chemokine is MIP-3 α , the physiology is pulmonary physiology, or the cell is an eosinophil.

DESCRIPTION OF THE DRAWINGS

Figures 1A-1B show chemotactic properties of mTECK recombinant protein. Fig. 1A shows migration of mouse thymocytes to recombinant mTECK and effect of pertussis toxin. Chemotaxis assays were performed as described. Recombinant mouse lymphotactin was used as a positive control. Data are expressed as the mean of cell counts obtained from three separate experiments in duplicate \pm SEM. In one experiment, cells were pre incubated 1 h with 10 ng/ml pertussis toxin (PTX) prior to the assay. Fig. 1B shows migration of other leukocyte subsets to recombinant mTECK. Mouse splenic dendritic cells and mouse activated macrophages were obtained. THP-1 human monocytic cells were used without or with a 16 h activation with IFN- γ . Results are obtained as the mean of the chemotactic index from three separate experiments per cell type in duplicate \pm SD. The number of cells migrating to medium alone was greater than 40 cells per 5 high power fields in each experiment. Recombinant MIP-1 α was used as a positive control.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

OUTLINE

- 5 I. General
- II. Purified Chemokines, Receptors
 - A. physical properties
 - B. biological properties
- 10 III. Physical Variants
 - A. sequence variants, fragments
 - B. post-translational variants
 - 1. glycosylation
 - 2. others
- 15 IV. Functional Variants
 - A. analogs; fragments
 - 1. agonists
 - 2. antagonists
 - B. mimetics
 - 1. protein
 - 2. chemicals
 - C. species variants
- 20 V. Antibodies
 - A. polyclonal
 - B. monoclonal
 - 25 C. fragments, binding compositions
- VI. Nucleic Acids
 - A. natural isolates; methods
 - B. synthetic genes
 - C. methods to isolate
- 30 VII. Making Chemokines, Receptors; Mimetics
 - A. recombinant methods
 - B. synthetic methods
 - C. natural purification
- VIII. Uses
 - 35 A. diagnostic
 - B. therapeutic
- IX. Kits
 - A. nucleic acid reagents
 - B. protein reagents
 - 40 C. antibody reagents
- X. Receptors

I. General

45 The present invention provides DNA sequences encoding various mammalian proteins which exhibit structural properties characteristic of a chemotactic cytokine, or chemokine. Other embodiments are directed to chemokine receptors. See, e.g., Lodi, et al. (1994) Science 263:1762-1767; Gronenborn and Clore (1991) Protein Engineering 4:263-269; Miller and Kranger (1992) Proc. Nat'l Acad. Sci. USA 89:2950-2954; Matsushima and

Oppenheim (1989) Cytokine 1:2-13; Stoeckle and Baker (1990) New Biol. 2:313-323; Oppenheim, et al. (1991) Ann. Rev. Immunol. 9:617-648; Schall (1991) Cytokine 3:165-183; and The Cytokine Handbook Academic Press, NY.

5 Mouse and human embodiments are described herein.

Chemokines play an important role in immune and inflammatory responses by inducing migration and adhesion of leukocytes. These small secreted molecules are a growing superfamily of 8-14 kDa proteins
10 characterized by a conserved four cysteine motif. See, e.g., Schall (1991) Cytokine 3:165-183; and Thomson (ed.) The Cytokine Handbook Academic Press, NY. Chemokines are secreted by activated leukocytes and act as a chemoattractant for a variety of cells which are
15 involved in inflammation. Besides chemoattractant properties, chemokines have been shown to induce other biological responses, e.g., modulation of second messenger levels such as Ca^{++} ; inositol phosphate pool changes (see, e.g., Berridge (1993) Nature 361:315-325
20 or Billah and Anthes (1990) Biochem. J. 269:281-291); cellular morphology modification responses; phosphoinositide lipid turnover; possible antiviral responses; and others. Thus, the chemokines provided herein may, alone or in combination with other
25 therapeutic reagents, have advantageous combination effects.

Moreover, there are reasons to suggest that chemokines may have effects on other cell types, e.g., attraction or activation of monocytes, dendritic cells,
30 T cells, eosinophils, and/or perhaps on basophils and/or neutrophils. They may also have chemoattractive effects on various neural cells including, e.g., dorsal root ganglia neurons in the peripheral nervous system and/or central nervous system neurons.

35 Membrane proteins which contain seven transmembrane segments have been characterized as G-protein coupled receptors. Many of these receptors have been characterized as receptors for chemokines, based in part on structural features. Chemokine receptors are

important in the signal transduction mechanisms mediated by the chemokines. They are useful markers for distinguishing cell populations, and have been implicated as specific receptors for retroviral
5 infections.

The chemokine superfamily was classically divided into two groups exhibiting characteristic structural motifs, the Cys-X-Cys (C-X-C) and Cys-Cys (C-C) families. These were distinguished on the basis of a
10 single amino acid insertion between the NH-proximal pair of cysteine residues and sequence similarity. Typically, the C-X-C chemokines, i.e., IL-8 and MGSA/Gro- α act on neutrophils but not on monocytes, whereas the C-C chemokines, i.e., MIP-1 α and RANTES,
15 are potent chemoattractants for monocytes and lymphocytes but not neutrophils. See, e.g., Miller, et al. (1992) Crit. Rev. Immunol. 12:17-46. A recently isolated chemokine, lymphotactin, does not belong to either group and may constitute a first member of a
20 third chemokine family, the C family. Lymphotactin does not have a characteristic CC or CXC motif, and acts on lymphocytes but not neutrophils and monocytes. See, e.g., Kelner et al. (1994) Science 266:1395-1399. This chemokine defines a new C-C chemokine family. Even more
25 recently, another chemokine exhibiting a CX3C motif has been identified, which establishes a fourth structural class.

The present invention provides additional chemokine reagents, e.g., nucleic acids, proteins and peptides,
30 antibodies, etc., related to the newly discovered respective chemokines designated TECK; MIP-3 α , and MIP-3 β .

In other embodiments, the invention provides two genes encoding novel 7-transmembrane (7-TM) receptors,
35 presumably G-protein coupled receptors and likely chemokine receptors. These 7-TM receptors are hypothesized to be chemokine receptors and have been designated DC CR and M/DC CR. Their ligands have not yet specifically been completely identified. However,

the receptors exhibit structural features typical of known chemokine receptors, e.g., 7 transmembrane spanning structures. They may exhibit properties of binding many different cytokines at varying specificities (shared or promiscuous binding specificity) or may exhibit high affinity for one (specific) or a subset (shared) of chemokines.

The described chemokines and receptors should be important for mediating various aspects of cellular, organ, tissue, or organismal physiology or development.

II. Purified chemokines, receptors

Mouse and human Thymus Expressed ChemoKine (TECK) nucleotide and amino acid sequences are shown in Table 1. Nucleotide and amino acid sequences of another novel chemokine, from human, designated MIP-3 α are provided in Table 2. Nucleotide and derived amino acid sequences of a third novel chemokine, from human, designated MIP-3 β are shown in Table 3. Generic descriptions of physical properties of polypeptides, nucleic acids, and antibodies where directed to one embodiment clearly are generally applicable to other chemokines or receptors described herein.

The nucleotide and amino acid sequences of a novel chemokine receptor found on dendritic cells (DC), from human, and designated DC CR, are provided in Table 4. The nucleotide and amino acid sequences of another novel chemokine receptor found on macrophages and dendritic cells, from human, and designated M/DC CR, are provided in Table 5.

These amino acid sequences, provided amino to carboxy, are important in providing sequence information on the chemokine ligand or receptor, allowing for distinguishing the protein from other proteins. Moreover, the sequences allow preparation of peptides to generate antibodies to recognize and distinguish such segments, and allow preparation of oligonucleotide probes, both of which are strategies for isolation, e.g., cloning, of genes encoding such sequences, or

related sequences, e.g., natural polymorphic or other variants. Similarities of the chemokines have been observed with other cytokines. See, e.g., Bosenberg, et al. (1992) Cell 71:1157-1165; Huang, et. al. (1992) 5 Molecular Biology of the Cell 3:349-362; and Pandiella, et al. (1992) J. Biol. Chem. 267:24028-24033. Likewise for the receptors.

Table 1: Nucleotide sequence (5' to 3') of TECK from mouse and the corresponding amino acid sequence (amino to carboxy). Signal sequence probably runs as shown between Ala and Gln, see SEQ ID NO: 1 and 2. Human sequences are SEQ ID NO: 3 and 4.

5	1	AGGCTACAAGCAGGCACCAGCTCTCAGGACCAGAAAGGCATTGGTGGCCCCCTTAAACCT	60
	61	TCAGGTATCTGGAGAGGAGATCTAACCTTCACTATGAAACTGTGGCTTTTTCCTGCCTG	120
10	1	MetLysLeuTrpLeuPheAlaCysLeu	9
	121	GTTGCCTGTTTTGTTGGGGCCTGGATGCCGTTGTCCATGCCCAAGGTGCCTTTGAAGAC	180
	10	ValAlaCysPheValGlyAlaTrpMetProValValHisAlaGlnGlyAlaPheGluAsp	29
15	181	TGCTGCCTGGGTTACCAGCACAGGATCAAATGGAATGTTCTCCGGCATGCTAGGAATTAT	240
	30	CysCysLeuGlyTyrGlnHisArgIleLysTrpAsnValLeuArgHisAlaArgAsnTyr	49
	241	CACCAGCAGGAAGTGAGTGGAAGCTGCAACCTACGTGCTGTGAGATTCTACTTCCGCCAG	300
20	50	HisGlnGlnGluValSerGlySerCysAsnLeuArgAlaValArgPheTyrPheArgGln	69
	301	AAAGTAGTGTGTGGGAATCCAGAGGACATGAATGTGAAGAGGGCGATAAGAATCTTGACA	360
	70	LysValValCysGlyAsnProGluAspMetAsnValLysArgAlaIleArgIleLeuThr	89
25	361	GCTAGGAAAAGGCTAGTCCACTGGAAGAGCGCCTCAGACTCTCAGACTGAAAGGAAGAAG	420
	90	AlaArgLysArgLeuValHisTrpLysSerAlaSerAspSerGlnThrGluArgLysLys	109
	421	TCAAACCATATGAAGTCCAAGGTGGAGAACCCCAACAGTACAAGCGTGAGGAGTGCCACC	480
	110	SerAsnHisMetLysSerLysValGluAsnProAsnSerThrSerValArgSerAlaThr	129
30	481	CTAGGTCATCCCAGGATGGTGATGATGCCCAGAAAGACCAACAATTAAGTTAATTACTCA	540
	130	LeuGlyHisProArgMetValMetMetProArgLysThrAsnAsnEnd	144
	541	GAGTAAGCACCAGCTGGAGGATGGGCGGAGTCTGCTGAAGTGCTGTCTTCTAGGCATGCC	600
35	601	AGTGCCAATGAACTCACTGAAGCTACAGTTTCCTGTACAAGACCAGACCCACCAACGTCT	660
	661	CAGCATGTACGAGGAAGGAAGTACTGCGCTAAAGGCCCTCCCACTCACCAAGGAGCTATT	720
	721	GGCTATTGATGATTGCTGAGGGAAGGGAGTAATTTTTTTTCTCTTTCTGAAGTGTGACTT	780
40	781	GAGTAAATTGCCCATAGTTTCAGTATATAATCCCCAACCTGTGCTCAGGCAAGCAACCCTA	840
	841	ATTAAATGCAATAGCCACATACAAAAGAAGAGGATATGAATAGTTTGGTAGGAGGGGCTT	900
45	901	GTTAGGAAGAAGACATTAACAGGAGAGAGAGGAGCGAGAGGATAGTGAGTGTGTGAGAGT	960
	961	GCCTGCACGTGTGAAATGGTCAAAGAATTAATAAATAAATAAAGCTATTAAAAA	
	1020		
50	1021	GTAAAAAAATAAAA 1034	

Table 1 (continued):

human Teck cDNA (see SEQ ID NO: 3); signal sequence cleavage is probably between about Thr and Gln. Hu TECK protein sequence (see SEQ ID NO: 4).

5	TCGACCCACG CGTCCGCTTG GCCTACAGCC CGGCGGGCAT CAGCTCCCTT GACCCAGTGG	60
	ATATCGGTGG CCCC GTTATT CGTCCAGGTG CCCAGGGAGG AGGACCCGCC TGCAGC	116
10	ATG AAC CTG TGG CTC CTG GCC TGC CTG GTG GCC GGC TTC CTG GGA GCC Met Asn Leu Trp Leu Leu Ala Cys Leu Val Ala Gly Phe Leu Gly Ala -23 -20 -15 -10	164
15	TGG GCC CCC GCT GTC CAC ACC CAA GGT GTC TTT GAG GAC TGC TGC CTG Trp Ala Pro Ala Val His Thr Gln Gly Val Phe Glu Asp Cys Cys Leu -5 1 5	212
20	GCC TAC CAC TAC CCC ATT GGG TGG GCT GTG CTC CGG CGC GCC TGG ACT Ala Tyr His Tyr Pro Ile Gly Trp Ala Val Leu Arg Arg Ala Trp Thr 10 15 20 25	260
25	TAC CGG ATC CAG GAG GTG AGC GGG AGC TGC AAT CTG CCT GCT GCG ATA Tyr Arg Ile Gln Glu Val Ser Gly Ser Cys Asn Leu Pro Ala Ala Ile 30 35 40	308
30	TTC TAC CTC CCC AAG AGA CAC AGG AAG GTG TGT GGG AAC CCC AAA AGC Phe Tyr Leu Pro Lys Arg His Arg Lys Val Cys Gly Asn Pro Lys Ser 45 50 55	356
35	AGG GAG GTG CAG AGA GCC ATG AAG CTC CTG GAT GCT CGA AAT AAG GTT Arg Glu Val Gln Arg Ala Met Lys Leu Leu Asp Ala Asn Lys Val 60 65 70	404
40	TTT GCA AAG CTC CAC CAC AAC ATG CAG ACC TTC CAA GCA GGC CCT CAT Phe Ala Lys Leu His His Asn Met Gln Thr Phe Gln Ala Gly Pro His 75 80 85	452
45	GCT GTA AAG AAG TTG AGT TCT GGA AAC TCC AAG TTA TCA TCA TCC AAG Ala Val Lys Lys Leu Ser Ser Gly Asn Ser Lys Leu Ser Ser Ser Lys 90 95 100 105	500
50	TTT AGC AAT CCC ATC AGC AGC AGC AAG AGG AAT GTC TCC CTC CTG ATA Phe Ser Asn Pro Ile Ser Ser Ser Lys Arg Asn Val Ser Leu Leu Ile 110 115 120	548
55	TCA GCT AAT TCA GGA CTG TGAGCCGGCT CATTTCTGGG CTCCATCGGC Ser Ala Asn Ser Gly Leu 125	596
60	ACAGGAGGGG CCGGATCTTT CTCCGATAAA ACCGTCGCCC TACAGACCCA GCTGTCCCCA CGCCTCTGTC TTTTGGGTCA AGTCTTAATC CCTGCACCTG AGTTGGTCCT CCCTCTGCAC CCCCACCACC TCCTGCCCCG CTGGCAACTG GAAAGAAGGA GTTGGCCTGA TTTTAACCTT TTGCCGCTCC GGGGAACAGC ACAATCCTGG GCAGCCAGTG GCTCTTGTAG AGAAACTTA GGATACCTCT CTCACCTTCT GTTTCTTGCC GTCCACCCCG GGCCATGCCA GTGTGTCTC	656 716 776 836 896

Table 1 (continued):

	TGGGTCCCCT CCAAAAATCT GGTCATTCAA GGATCCCCTC CCAAGGCTAT GCTTTTCTAT	956
5	AACTTTTAAA TAAACCTTGG GGGGTGAATG GAATAAAAAA AAAAAAAAAA AAAAAA	1012

10 Table 2: Nucleotide sequence (5' to 3') of MIP-3 α from human and the corresponding amino acid sequence (amino to carboxy), see SEQ ID NO: 5 and 6 and GenBank Accession U77035.

	ATG TGC TGT ACC AAG AGT TTG CTC CTG GCT GCT TTG ATG TCA GTG CTG	48
15	Met Cys Cys Thr Lys Ser Leu Leu Leu Ala Ala Leu Met Ser Val Leu	
	-26 -25 -20 -15	
	CTA CTC CAC CTC TGC GGC GAA TCA GAA GCA GCA AGC AAC TTT GAC TGC	96
	Leu Leu His Leu Cys Gly Glu Ser Glu Ala Ala Ser Asn Phe Asp Cys	
	-10 -5 1 5	
20	TGT CTT GGA TAC ACA GAC CGT ATT CTT CAT CCT AAA TTT ATT GTG GGC	144
	Cys Leu Gly Tyr Thr Asp Arg Ile Leu His Pro Lys Phe Ile Val Gly	
	10 15 20	
25	TTC ACA CGG CAG CTG GCC AAT GAA GGC TGT GAC ATC AAT GCT ATC ATC	192
	Phe Thr Arg Gln Leu Ala Asn Glu Gly Cys Asp Ile Asn Ala Ile Ile	
	25 30 35	
30	TTT CAC ACA AAG AAA AAG TTG TCT GTG TGC GCA AAT CCA AAA CAG ACT	240
	Phe His Thr Lys Lys Lys Leu Ser Val Cys Ala Asn Pro Lys Gln Thr	
	40 45 50	
35	TGG GTG AAA TAT ATT GTG CGT CTC CTC AGT AAA AAA GTC AAG AAC ATG	288
	Trp Val Lys Tyr Ile Val Arg Leu Leu Ser Lys Lys Val Lys Asn Met	
	55 60 65 70	
	TAAAACTGT GGCTTTTCTG GAATGGAATT GGACATAGCC CAAGAACAGA AGAACCTTG	348
40	CTGGGGTTGG AGGTTTCACT TGCACATCAT GGAGGGTTTA GTGCTTATCT AATTTGTGCC	408
	TCACTGGACT TGTCCAATTA ATGAAGTTGA TTCATATTGC ATCATAGTTT GCTTTGTTTA	468
	AGCATCACAT TAAAGTTAAA CTGTATTTTA TGTTATTTAT AGCTGTAGGT TTTCTGTGTT	528
45	TAGCTATTTA ATACTAATTT TCCATAAGCT ATTTTGGTTT AGTGCAAAGT ATAAAATTAT	588
	ATTTGGGGGG GAATAAGATT ATATGGACTT TTTTGCAAGC AACAAGCTAT TTTTAAAAA	648
50	AAACTATTTA ACATTCTTTT GTTTATATTG TTTTGTCTCC TAAATTGTTG TAATTGCATT	708
	ATAAAATAAG AAAAATATTA ATAAGACAAA TATTGAAAAT AAAGAAACAA AAAGTTAAAA	768
	AAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAA	801

55

Table 3: Nucleotide sequence (5' to 3') of MIP-3 β from human and the corresponding amino acid sequence (amino to carboxy), see SEQ ID NO: 7 and 8, and GenBank Accession U77180. Signal sequence cleavage is about between Ser and Gly.

5	1	GGCACGAGCGGCACGAGCATCACTCACACCTTGCATTTACCCCTGCATCCCAGTCGCCC	60
	61	TGCAGCCTCACACAGATCCTGCACACACCCAGACAGCTGGCGCTCACACATTCACCGTTG	120
10	121	GCCTGCCTCTGTTACCCCTCCATGGCCCTGCTACTGGCCCTCAGCCTGCTGGTTCTCTGG	180
	1	MetAlaLeuLeuLeuAlaLeuSerLeuLeuValLeuTrp	13
	181	ACTTCCCCAGCCCCAACTCTGAGTGGCACCAATGATGCTGAAGACTGCTGCCTGTCTGTG	240
15	14	ThrSerProAlaProThrLeuSerGlyThrAsnAspAlaGluAspCysCysLeuSerVal	33
	241	ACCCAGAAACCCATCCCTGGGTACATCGTGAGGAACTTCCACTACCTTCTCATCAAGGAT	300
	34	ThrGlnLysProIleProGlyTyrIleValArgAsnPheHisTyrLeuLeuIleLysAsp	53
	301	GGCTGCAGGGTGCCTGCTGTAGTGTTACCCACACTGAGGGGCCGCCAGCTCTGTGCACCC	360
20	54	GlyCysArgValProAlaValValPheThrThrLeuArgGlyArgGlnLeuCysAlaPro	73
	361	CCAGACCAGCCCTGGGTAGAACGCATCATCCAGAGACTGCAGAGGACCTCAGCCAAGATG	420
	74	ProAspGlnProTrpValGluArgIleIleGlnArgLeuGlnArgThrSerAlaLysMet	93
25	421	AAGCGCCGCAGCAGTTAACCTATGACCGTGCAGAGGGAGCCCGGAGTCCGAGTCAAGCAT	480
	94	LysArgArgSerSerEnd	98
	481	TGTGAATTATTACCTAACCTGGGGAACCGAGGACCAGAAGGAAGGACCAGGCTTCCAGCT	540
30	541	CCTCTGCACCAGACCTGACCAGCCAGGACAGGGCCTGGGGTGTGTGTGAGTGTGAGTGTG	600
	601	AGCGAGAGGGTGAGTGTGGTCTAGAGTAAAGCTGCTCCACCCCCAGATTGCAATGCTACC	660
35	661	AATAAAGCCGCCTGGTGTTTACAATAAAAAAAAAAAAAA	699

Table 4: Nucleotide sequence (5' to 3') of chemokine receptor, DC CR, from human and the corresponding amino acid sequence (amino to carboxy), see SEQ ID NO: 9 and 10. Nucleotide 579 may be A, C, G, or T, and the codon may code for His or Gln.

5	1	ATGTTTTCTCGACTCCAGTGAAGATTATTTTGTGTCAAGTCAATACTTCATATTACTCAGTTG	60
	1	MetPheSerThrProValLysIleIleLeuCysGlnSerIleLeuHisIleThrGlnLeu	20
10	61	ATTCTGAGATGTTACTGTGCTCCTTGAGGAGGTCAGGCAGTTCTCCAGGCTATTTGTAC	120
	21	IleLeuArgCysTyrCysAlaProCysArgArgSerGlySerSerProGlyTyrLeuTyr	40
15	121	CGAATTGCCTACTCCTTGATCTGTGTTCTTGGCCTCCTGGGGAATATTCTGGTGGTGATC	180
	41	ArgIleAlaTyrSerLeuIleCysValLeuGlyLeuLeuGlyAsnIleLeuValValIle	60
20	181	ACCTTTGCTTTTTTATAAGAAGGCCAGGTCTATGACAGACGTCTATCTCTTGAACATGGCC	240
	61	ThrPheAlaPheTyrLysLysAlaArgSerMetThrAspValTyrLeuLeuAsnMetAla	80
25	241	ATTGCAGACATCCTCTTTGTTCTTACTCTCCCATTCTGGGCAGTGAGTCATGCCACTGGT	300
	81	IleAlaAspIleLeuPheValLeuThrLeuProPheTrpAlaValSerHisAlaThrGly	100
30	301	GCGTGGGTTTTTCAGCAATGCCACGTGCAAGTTGCTAAAAGGCATCTATGCCATCAACTTT	360
	101	AlaTrpValPheSerAsnAlaThrCysLysLeuLeuLysGlyIleTyrAlaIleAsnPhe	120
35	361	AACTGCGGGATGCTGCTCCTGACTTGCAATTAGCATGGACCGGTACATCGCCATTGTACAG	420
	121	AsnCysGlyMetLeuLeuLeuThrCysIleSerMetAspArgTyrIleAlaIleValGln	140
40	421	GCGACTAAGTCATTCCGGCTCCGATCCAGAACACTACCGCGCAGCAAAATCATCTGCCTT	480
	141	AlaThrLysSerPheArgLeuArgSerArgThrLeuProArgSerLysIleIleCysLeu	160
45	481	GTTGTGTGGGGGCTGTCAGTCATCATCTCCAGCTCAACTTTTGTCTTCAACCAAAATAC	540
	161	ValValTrpGlyLeuSerValIleIleSerSerSerThrPheValPheAsnGlnLysTyr	180
50	541	AACACCCAAGGCAGCGATGTCTGTGAACCAAGTACCAAACTGTCTCGGAGCCCATCAGG	600
	181	AsnThrGlnGlySerAspValCysGluProLysTyrGlnThrValSerGluProIleArg	200
55	601	TGGAAGCTGCTGATGTTGGGGCTTGAGCTACTCTTTGGTTTCTTTATCCCTTTGATGTTT	660
	201	TrpLysLeuLeuMetLeuGlyLeuGluLeuLeuPheGlyPhePheIleProLeuMetPhe	220
60	661	ATGATATTTTGTACACGTTTATTGTCAAAACCTTGGTGCAAGCTCAGAATTCTAAAAGG	720
	221	MetIlePheCysTyrThrPheIleValLysThrLeuValGlnAlaGlnAsnSerLysArg	240
65	721	CACAAAGCCATCCGTGTAATCATAGCTGTGGTGCTTGTGTTTCTGGCTTGTCAGATTCT	780
	241	HisLysAlaIleArgValIleIleAlaValValLeuValPheLeuAlaCysGlnIlePro	260
70	781	CATAACATGGTCCTGCTTGTGACGGCTGCTAATTTGGGTAAAATGAACCGATCCTGCCAG	840
	261	HisAsnMetValLeuLeuValThrAlaAlaAsnLeuGlyLysMetAsnArgSerCysGln	280
75	841	AGCGAAAAGCTAATTGGCTATACGAAAAGTGTACAGAAAGTCCTGGCTTTCCTGCACTGC	900
	281	SerGluLysLeuIleGlyTyrThrLysThrValThrGluValLeuAlaPheLeuHisCys	300
80	901	TGCCTGAACCCCTGTGCTCTACGCTTTTATTGGGCAGAAAGTTCAGAACTACTTTCTGAAG	960
	301	CysLeuAsnProValLeuTyrAlaPheIleGlyGlnLysPheArgAsnTyrPheLeuLys	320
85	961	ATCTTGAAGGACCTGTGGTGTGTGAGAAGGAAGTACAAGTCCTCAGGCTTCTCCTGTGCC	
	1020		
90	321	IleLeuLysAspLeuTrpCysValArgArgLysTyrLysSerSerGlyPheSerCysAla	340
	1021	GGGAGGTACTCAGAAAACATTTCTCGGCAGACCAGTGAGACCGCAGATAACGACAATGCC	
95	1080		
	341	GlyArgTyrSerGluAsnIleSerArgGlnThrSerGluThrAlaAspAsnAspAsnAla	360

Table 4 (continued)

1081 TCGTCCTTCACTATGTGATAGAAAGCTGAGTCTCCCTAA 1119
 361 SerSerPheThrMetEnd 365

5

Table 5: Nucleotide sequence (5' to 3') of chemokine receptor, M/DC CR, from human and the corresponding amino acid sequence (amino to carboxy), see SEQ ID NO: 11 and 12.

10

	1	GAGGAAGCTGCTTCGGGGGGTGAGCAAACCTTTTAAAAATGCAGAAATTATGATCTACACC	60
		MetIleTyrThr	4
	61	CGTTTCTTAAAAGGCAGTCTGAAGATGGCCAATTACACGCTGGCACCAGAGGATGAATAT	120
15	5	ArgPheLeuLysGlySerLeuLysMetAlaAsnTyrThrLeuAlaProGluAspGluTyr	24
	121	GATGTCCTCATAGAAGGTGAAGTGGAGAGCGATGAGGCAGAGCAATGTGACAAGTATGAC	180
	25	AspValLeuIleGluGlyGluLeuGluSerAspGluAlaGluGlnCysAspLysTyrAsp	44
20	181	GCCCAGGCACTCTCAGCCCAGCTGGTGCCATCACTCTGCTCTGCTGTGTTTGTGATCGGT	240
	45	AlaGlnAlaLeuSerAlaGlnLeuValProSerLeuCysSerAlaValPheValIleGly	64
	241	GTCCTGGACAATCTCCTGGTTGTGCTTATCCTGGTAAAAATATAAAGGACTCAAACGCGTG	300
25	65	ValLeuAspAsnLeuLeuValValLeuIleLeuValLysTyrLysGlyLeuLysArgVal	84
	301	GAAAAATATCTATCTTCTAAACTTGGCAGTTTCTAACTTGTGTTTCTTGCTTACCCTGCCC	360
	85	GluAsnIleTyrLeuLeuAsnLeuAlaValSerAsnLeuCysPheLeuLeuThrLeuPro	104
	361	TTCTGGGCTCATGCTGGGGGCGATCCCATGTGTAAAATTCTCATTGGACTGTACTTCGTG	420
30	105	PheTrpAlaHisAlaGlyGlyAspProMetCysLysIleLeuIleGlyLeuTyrPheVal	124
	421	GGCCTGTACAGTGAGACATTTTTCAATTGCCTTCTGACTGTGCAAAGGTACCTAGTGT	480
	125	GlyLeuTyrSerGluThrPhePheAsnCysLeuLeuThrValGlnArgTyrLeuValPhe	144
35	481	TTGCACAAGGGCAACTTTTTCTCAGCCAGGAGGAGGGTGCCCTGTGGCATCATTACAAGT	540
	145	LeuHisLysGlyAsnPhePheSerAlaArgArgArgValProCysGlyIleIleThrSer	164
	541	GTCCTGGCATGGGTAACAGCCATTCTGGCCACTTTGCCTGAATTCGTGGTTTATAAACCT	600
40	165	ValLeuAlaTrpValThrAlaIleLeuAlaThrLeuProGluPheValValTyrLysPro	184
	601	CAGATGGAAGACCAGAAATACAAGTGTGCATTTAGCAGAACTCCCTTCCTGCCAGCTGAT	660
	185	GlnMetGluAspGlnLysTyrLysCysAlaPheSerArgThrProPheLeuProAlaAsp	204
	661	GAGACATTCTGGAAGCATTCTTCTGACTTTAAAAATGAACATTCGGTTCTTGTCTCTCCC	720
45	205	GluThrPheTrpLysHisPheLeuThrLeuLysMetAsnIleSerValLeuValLeuPro	224
	721	CTATTTATTTTACATTTCTCTATGTGCAAATGAGAAAAACACTAAGGTTTCAAGGAGCAG	780
	225	LeuPheIlePheThrPheLeuTyrValGlnMetArgLysThrLeuArgPheArgGluGln	244
50	781	AGGTATAGCCTTTTCAAGCTTGTGTTTGGCCGTAATGGTAGTCTTCCTTCTGATGTGGGCG	840
	245	ArgTyrSerLeuPheLysLeuValPheAlaValMetValValPheLeuLeuMetTrpAla	264
	841	CCCTACAATATTGCATTTTCTGTCCACTTTCAAAGAACTTCTCCCTGAGTGACTGC	900
55	265	ProTyrAsnIleAlaPhePheLeuSerThrPheLysGluHisPheSerLeuSerAspCys	284
	901	AAGAGCAGCTACAATCTGGACAAAAGTGTTCACATCACTAAACTCATCGCCACCACCCAC	960
	285	LysSerSerTyrAsnLeuAspLysSerValHisIleThrLysLeuIleAlaThrThrHis	304
	961	TGCTGCATCAACCCTCTCCTGTATGCGTTTCTTGATGGGACATTAGCAAATACCTCTGC	
60	1020		
	305	CysCysIleAsnProLeuLeuTyrAlaPheLeuAspGlyThrPheSerLysTyrLeuCys	324

1021	CGCTGTTTCCATCTGCGTAGTAACACCCCACTTCAACCCAGGGGGCAGTCTGCACAAGGC	
1080		
325	ArgCysPheHisLeuArgSerAsnThrProLeuGlnProArgGlyGlnSerAlaGlnGly	344
1081	ACATCGAGGGAAGAACCTGACCATTCCACCGAAGTGTAAGTATGATCCACCAAATGCAA	
1140		
345	ThrSerArgGluGluProAspHisSerThrGluValEnd	356
1141	GAAGAATAAACATGGATTTTCATCTTTCTGCATTATTTTCATGTAAATTTTCTACACATTT	
1200		
1201	GTATACAAAATCGGATACAGGAAGAAAAGGGAGAGGTGAGCTAACATTTGCTAAGCACTG	
1260		
1261	AATTTGTCTCAGGCACCGTGCAAGGCTCTTTACAAACGTGAGCTCCTTCGCCTCCTACCA	
1320		
1321	CTTGTCCATAGTGTGGATAGGACTAGTCTCATTTCTCTGAGAAGAAAATAAGGCGCGGA	
1380		
1381	AATTTGTCTAAGATCACATAACTAGGAAGTGGCAGAACTGATTCTCCAGCCCTGGTAGCA	
1440		
1441	TTTGCTCAGAGCCTACGCTTGGTCCAGAACATCAAACCTCCAAACCCTGGGGACAAACGAC	
1500		
1501	ATGAAATAAATGTATTTTAAACATATAAAAAAAAAAAAAAAAAAAAAA	
1547		

40	M/DC CR	MIYTRFLKGSLKMANYTALAPEDVEYDVLIEGELESDEAEQCDKYDAQAALS
	C-C CKR-1	METPNTTETYDTTTEFDYGDATPCQKVNERAFG
	C-C CKR-2	MLSTSRSRFIRNTNESGEEVTTFFDYDYGAPCHKFDVKQIG
	C-C CKR-3	MTTSLDTVETFGTTSYDDVGLLCEKADTRALM
	C-C CKR-4	MNPTDIADTTLDESIYSNYLYYESIPKPCTKEGIKAFG
		* * . .
45	M/DC CR	AQLVPSLCSAVFVIGVLDNLLVVLILVKYKGLKRVENIYLLNLAVSNLCF
	C-C CKR-1	AQLLPPLYSLVFLVIGLVGNILVVLVVLVQYKRLKNMTSIYLLNLAISDLLF
	C-C CKR-2	AQLLPPLYSLVFIFFGVGNMLVVLILINCKKLKCLTDIYLLNLAISDLLF
	C-C CKR-3	AQFVPPLYSLVFTVGLLGNVVVMILIKYRRLRIMTNIYLLNLAISDLLF
50	C-C CKR-4	ELFLPPLYSLVFLVFGLLGNSVVVLVLFKYKRLRSMTDVYLLNLAISDLLF
		. . * . * * * * . . * . . . * * * * . . * *
	M/DC CR	LLTLPFWAHAG-----GDPMCKILIGLYFVGLYSETFFNCLLTVQRYL
	C-C CKR-1	LFTLPFWIDYKLKDDWVFGDAMCKILSGFYTGlySEIFFIILLTIDRYL
55	C-C CKR-2	LITLPLWAH-SANEWVFGNAMCKLFTGLYHIGYFGGIFFIILLTIDRYL
	C-C CKR-3	LVTLPFWIHVYVRGHNWVFGHGMCKLLSGFYHTGLYSEIFFIILLTIDRYL
	C-C CKR-4	VFSLPFWGYA-ADQWVFGGLGLCKMISWMYLVGFYSGIFFVMLMSIDRYL
		. . * . * * * . . . * * . . * * * . . . * *
60	M/DC CR	VFLHKGNNFFSAR-RRVPCGIITSVLAWVTAILATLPEFVVYKPQMEDQKY
	C-C CKR-1	AIVH--AVFALRARTVTFGVITSIIIWALAILASMPGLYFSKTQWEFTHH
	C-C CKR-2	AIVH--AVFALKARTVTFGVITSVITWLVAVFASVPGIIFTKCQKEDSVY
	C-C CKR-3	AIVH--AVFALRARTVTFGVITSIVTWGLAVLAALPEFIFYETEELFEET
	C-C CKR-4	AIVH--AVFSLRARTLTYGVITSLATWSVAVFASLPGFLFSTCYTERNHT
65		* * * * * * * * * * * *

Table 6 (continued)

	M/DC CR	KCAFSRTPFLPADETF-WKHFLTLMKNISVLVLPLFIFFFLYVQMRKTL-
	C-C CKR-1	TCS----LHFPHESLREWKLFLQALKLNLFGLVLPPLLVMIICTGIIKILL
5	C-C CKR-2	VCG----PYFPR----GWNNFHTIMRNILGLVLPPLLIMVICYSGILKTLL
	C-C CKR-3	LCS----ALYPEDTVYSWRHFHTLRMTIFCLVLPPLLVMAICYTGIIKTLL
	C-C CKR-4	YCK----TKYSLNST-TWKVLSLSLEINILGLVIPLGIMLFCYSMIIRTLO
		* * * *
10	M/DC CR	--RFREQRYSLFKLVFVAVMVVFLLMWAPYNIAFFLSTFKEHFSLSDCCKSS
	C-C CKR-1	RRPNEKK-SKAVRLIFVIMIIFFLFWTPYNLTILISVFQDFLFTHCEQS
	C-C CKR-2	RCRNEKKRHRAVRVIFTIMIVYFLFWTPYNIVILLNTFQEFGLSNCEST
	C-C CKR-3	RCPSKKK-YKAIRLIFVIMAVFFIFWTPYNVAILLSSYSILFGNDCERS
	C-C CKR-4	HCKNEKK-NKAVKMIFAVVVLFLGFWTPYNIVLFLFETLVELEVLQDCTFE
15	 * * * *
	M/DC CR	YNLDKSVHITKLIATTHCCINPLLYAFLDGTFSKYLRCRCFH-----
	C-C CKR-1	RHLDLAVQVTEVIAIYTHCCVNPVIYAFVGERFRKYLRQLFH-RRVA----
	C-C CKR-2	SQLDQATQVTETLGMTHCCINPIIYAFVGEKFRSLFHIALG-CRIAPLQK
20	C-C CKR-3	KHLDLVMLVTEVIAIYSHCCMNPVIYAFVGERFRKYLRHFFH-RHLL----
	C-C CKR-4	RYLDYAIQATETLAFAVHCCCLNPVIYFFLGEKFRKYLRQLFKTCRGLFVLC
		** * * *
	M/DC CR	-----LRSNTPLQPRGQSAQGTSREEP--DHSTEV*
25	C-C CKR-1	-----VHLVKWLPFLSVDRLERVSSTSPSTGEHELSEA---GF*
	C-C CKR-2	PVCGGPGVRPGKNVKVTTQGLLDGRGKGKSIGRAPEASLQDKEGA*
	C-C CKR-3	-----MHLGRYIPFLPSEKLERTSSVSPSTAEPESI---VF*
	C-C CKR-4	QYCG-----LLQIYSAD-----TPSSSYTQSTMDHDLHDAL*
30		

As used herein, the term "TECK" shall encompass, when used in a protein context, a protein having mature mouse or human amino acid sequences, as shown in Table 1. The invention also embraces a polypeptide comprising a significant fragment of such protein. It also refers to a polypeptide which is a species counterpart, e.g., which exhibits similar biological function, and is more homologous in natural encoding sequence than other genes from that species. Typically, such chemokine will also interact with its specific binding components, e.g., receptor. These binding components, e.g., antibodies, typically bind to the chemokine with high affinity, e.g., at least about 100 nM, usually better than about 30 nM, preferably better than about 10 nM, and more preferably at better than about 3 nM. Homologous proteins would be found in mammalian species other than mouse, e.g., rats, dogs, cats, and primates. Non-mammalian species should also possess structurally or functionally related genes and proteins.

The term "polypeptide" as used herein includes a significant fragment or segment, and encompasses a

stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 12 amino acids, often at least 14 amino acids, more often at least 16 amino acids, typically at least
5 18 amino acids, more typically at least 20 amino acids, usually at least 22 amino acids, more usually at least 24 amino acids, preferably at least 26 amino acids, more preferably at least 28 amino acids, and, in particularly preferred embodiments, at least about 30 or more amino
10 acids, e.g., about 35, 40, 45, 50, 60, 75, 80, 100, 120, etc. Similar proteins will likely comprise a plurality of such segments. Such fragments may have ends which begin and/or end at virtually all positions, e.g., beginning at residues 1, 2, 3, etc., and ending at,
15 e.g., 69, 68, 67, 66, etc., in all combinations. Particularly interesting peptides have ends corresponding to structural domain boundaries. See, e.g., PHD and DSC programs, Rost and Sander (1994) Proteins 19:55-72; and King and Sternberg (1996) Protein
20 Science 5:2298-2310.

The term "binding composition" refers to molecules that bind with specificity to the respective chemokine or receptor, e.g., in a ligand-receptor type fashion or an antibody-antigen interaction. These compositions may
25 be compounds, e.g., proteins, which specifically associate with the chemokine or receptor, including natural physiologically relevant protein-protein interactions, either covalent or non-covalent. The binding composition may be a polymer, or another
30 chemical reagent. No implication as to whether the chemokine presents a concave or convex shape in its ligand-receptor interaction is represented, other than the interaction exhibit similar specificity, e.g., specific affinity. A functional analog may be a ligand
35 with structural modifications, or may be a wholly unrelated molecule, e.g., which has a molecular shape which interacts with the appropriate ligand binding determinants. The ligands may serve as agonists or antagonists of the receptor, see, e.g., Goodman, et al.

(eds.) (1990) Goodman & Gilman's: The Pharmacological Bases of Therapeutics (8th ed.), Pergamon Press.

Substantially pure means that the protein is free from other contaminating proteins, nucleic acids, and/or
5 other biologicals typically derived from the original source organism. Purity may be assayed by standard methods, and will ordinarily be at least about 40% pure, more ordinarily at least about 50% pure, generally at least about 60% pure, more generally at least about 70%
10 pure, often at least about 75% pure, more often at least about 80% pure, typically at least about 85% pure, more typically at least about 90% pure, preferably at least about 95% pure, more preferably at least about 98% pure, and in most preferred embodiments, at least 99% pure.
15 Analyses will typically be by weight, but may be by molar amounts.

Solubility of a polypeptide or fragment depends upon the environment and the polypeptide. Many parameters affect polypeptide solubility, including
20 temperature, electrolyte environment, size and molecular characteristics of the polypeptide, and nature of the solvent. Typically, the temperature at which the polypeptide is used ranges from about 4° C to about 65° C. Usually the temperature at use is greater than about
25 18° C and more usually greater than about 22° C. For diagnostic purposes, the temperature will usually be about room temperature or warmer, but less than the denaturation temperature of components in the assay. For therapeutic purposes, the temperature will usually
30 be body temperature, typically about 37° C for humans, though under certain situations the temperature may be raised or lowered in situ or in vitro.

The electrolytes will usually approximate in situ physiological conditions, but may be modified to higher
35 or lower ionic strength where advantageous. The actual ions may be modified, e.g., to conform to standard buffers used in physiological or analytical contexts.

The size and structure of the polypeptide should generally be in a substantially stable state, and

usually not in a denatured state. The polypeptide may be associated with other polypeptides in a quaternary structure, e.g., to confer solubility, or associated with lipids or detergents in a manner which approximates
5 natural lipid bilayer interactions.

The solvent will usually be a biologically compatible buffer, of a type used for preservation of biological activities, and will usually approximate a physiological solvent. Usually the solvent will have a
10 neutral pH, typically between about 5 and 10, and preferably about 7.5. On some occasions, a detergent will be added, typically a mild non-denaturing one, e.g., CHS or CHAPS, or a low enough concentration as to avoid significant disruption of structural or
15 physiological properties of the protein.

Solubility is reflected by sedimentation measured in Svedberg units, which are a measure of the sedimentation velocity of a molecule under particular conditions. The determination of the sedimentation
20 velocity was classically performed in an analytical ultracentrifuge, but is typically now performed in a standard ultracentrifuge. See, Freifelder (1982) Physical Biochemistry (2d ed.), W.H. Freeman; and Cantor and Schimmel (1980) Biophysical Chemistry, parts 1-3, W.H. Freeman & Co., San Francisco. As a crude
25 determination, a sample containing a putatively soluble polypeptide is spun in a standard full sized ultracentrifuge at about 50K rpm for about 10 minutes, and soluble molecules will remain in the supernatant. A
30 soluble particle or polypeptide will typically be less than about 30S, more typically less than about 15S, usually less than about 10S, more usually less than about 6S, and, in particular embodiments, preferably less than about 4S, and more preferably less than about
35 3S.

III. Physical Variants

This invention also encompasses proteins or peptides having substantial amino acid sequence homology

with the amino acid sequence of each respective chemokine or receptor. The variants include species or polymorphic variants.

Amino acid sequence homology, or sequence identity, is determined by optimizing residue matches, if necessary, by introducing gaps as required. This changes when considering conservative substitutions as matches. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Homologous amino acid sequences are typically intended to include natural allelic and interspecies variations in each respective protein sequence. Typical homologous proteins or peptides will have from 25-100% homology (if gaps can be introduced), to 50-100% homology (if conservative substitutions are included) with the amino acid sequence of the appropriate chemokine or receptor. Homology measures will be at least about 35%, generally at least 40%, more generally at least 45%, often at least 50%, more often at least 55%, typically at least 60%, more typically at least 65%, usually at least 70%, more usually at least 75%, preferably at least 80%, and more preferably at least 80%, and in particularly preferred embodiments, at least 85% or more. See also Needleham, et al. (1970) J. Mol. Biol. 48:443-453; Sankoff, et al. (1983) Chapter One in Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparison Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA; and the University of Wisconsin Genetics Computer Group, Madison, WI.

Each of the isolated chemokine or receptor DNAs can be readily modified by nucleotide substitutions, nucleotide deletions, nucleotide insertions, and inversions of nucleotide stretches. These modifications result in novel DNA sequences which encode these antigens, their derivatives, or proteins having similar

physiological, immunogenic, or antigenic activity. These modified sequences can be used to produce mutant antigens or to enhance expression. Enhanced expression may involve gene amplification, increased transcription, increased translation, and other mechanisms. Such mutant chemokine or receptor derivatives include predetermined or site-specific mutations of the respective protein or its fragments. "Mutant chemokine" encompasses a polypeptide otherwise falling within the homology definition of the chemokine as set forth above, but having an amino acid sequence which differs from that of the chemokine as found in nature, whether by way of deletion, substitution, or insertion. These include substitution levels from none, one, two, three, etc. In particular, "site specific mutant chemokine" generally includes proteins having significant homology with a ligand having sequences of Table 1 through 3, and as sharing various biological activities, e.g., antigenic or immunogenic, with those sequences, and in preferred embodiments contain most of the disclosed sequences. Similar concepts apply to the different chemokine protein embodiments, particularly those found in various warm blooded animals, e.g., mammals and birds. As stated before, it is emphasized that descriptions are generally meant to encompass the various chemokine proteins, not limited to the mouse or human embodiments specifically discussed. Similar concepts apply to the receptor embodiments.

Although site specific mutation sites are often predetermined, mutants need not be site specific. Chemokine mutagenesis can be conducted by making amino acid insertions or deletions. Substitutions, deletions, insertions, or combinations may be generated to arrive at a final construct. Insertions include amino- or carboxy- terminal fusions. Random mutagenesis can be conducted at a target codon and the expressed mutants can then be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a known sequence are well known in the

art, e.g., by M13 primer mutagenesis or polymerase chain reaction (PCR) techniques. See also Sambrook, et al. (1989) and Ausubel, et al. (1987 and Supplements).

5 The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structure such as loops or hairpins.

10 The present invention also provides recombinant proteins, e.g., heterologous fusion proteins using segments from these proteins. A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of an immunoglobulin with a chemokine
15 or receptor polypeptide is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and exhibiting properties derived from each source peptide. A similar chimeric concept applies to heterologous
20 nucleic acid sequences.

In addition, new constructs may be made from combining similar functional domains from other proteins. For example, ligand-binding or other segments may be "swapped" between different new fusion
25 polypeptides or fragments. See, e.g., Cunningham, et al. (1989) Science 243:1330-1336; and O'Dowd, et al. (1988) J. Biol. Chem. 263:15985-15992. Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of
30 ligand-binding specificities and other functional domains.

The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A double
35 stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase

with an appropriate primer sequence, e.g., PCR techniques.

IV. Functional Variants

5 The blocking of physiological response to various
embodiments of these chemokines may result from the
inhibition of binding of the ligand to its receptor,
likely through competitive inhibition. Thus, in vitro
assays of the present invention will often use isolated
10 protein, membranes from cells expressing a recombinant
membrane associated chemokine, soluble fragments
comprising receptor binding segments of these ligands,
or fragments attached to solid phase substrates. These
assays will also allow for the diagnostic determination
15 of the effects of either binding segment mutations and
modifications, or ligand mutations and modifications,
e.g., ligand analogs.

 This invention also contemplates the use of
competitive drug screening assays, e.g., where
20 neutralizing antibodies to antigen or receptor fragments
compete with a test compound for binding to the protein.
In this manner, the antibodies can be used to detect the
presence of polypeptides which share one or more
antigenic binding sites of the ligand and can also be
25 used to occupy binding sites on the protein that might
otherwise interact with a receptor.

 Additionally, neutralizing antibodies against a
specific chemokine embodiment and soluble fragments of
the chemokine which contain a high affinity receptor
30 binding site, can be used to inhibit chemokine activity
in tissues, e.g., tissues experiencing abnormal
physiology.

 "Derivatives" of chemokine antigens include amino
acid sequence mutants, glycosylation variants, and
35 covalent or aggregate conjugates with other chemical
moieties. Covalent derivatives can be prepared by
linkage of functionalities to groups which are found in
chemokine amino acid side chains or at the N- or C-
termini, by means which are well known in the art.

These derivatives can include, without limitation, aliphatic esters or amides of the carboxyl terminus, or of residues containing carboxyl side chains, O-acyl derivatives of hydroxyl group-containing residues, and
5 N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g., lysine or arginine. Acyl groups are selected from the group of alkyl-moieties including C3 to C18 normal alkyl, thereby forming alkanoyl aroyl species. Covalent attachment to
10 carrier proteins may be important when immunogenic moieties are haptens.

In particular, glycosylation alterations are included, e.g., made by modifying the glycosylation patterns of a polypeptide during its synthesis and
15 processing, or in further processing steps. Particularly preferred means for accomplishing this are by exposing the polypeptide to glycosylating enzymes derived from cells which normally provide such processing, e.g., mammalian glycosylation enzymes.
20 Deglycosylation enzymes are also contemplated. Also embraced are versions of the same primary amino acid sequence which have other minor modifications, including phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

25 A major group of derivatives are covalent conjugates of the respective chemokine or receptor or fragments thereof with other proteins or polypeptides. These derivatives can be synthesized in recombinant culture such as N- or C-terminal fusions or by the use
30 of agents known in the art for their usefulness in cross-linking proteins through reactive side groups. Preferred chemokine derivatization sites with cross-linking agents are at free amino groups, carbohydrate moieties, and cysteine residues.

35 Fusion polypeptides between these chemokines and other homologous or heterologous proteins, e.g., other chemokines, are also provided. Many growth factors and cytokines are homodimeric entities, and a repeat construct may have various advantages, including

lessened susceptibility to proteolytic cleavage.

Moreover, many receptors require dimerization to transduce a signal, and various dimeric ligands or domain repeats can be desirable. Homologous

5 polypeptides may be fusions between different surface markers, resulting in, e.g., a hybrid protein exhibiting receptor binding specificity. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the
10 derivative proteins. Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with a segment or domain of a ligand, e.g., a receptor-binding segment, so that the presence or location of the fused ligand may be easily determined. See, e.g., Dull, et al., U.S.
15 Patent No. 4,859,609. Other gene fusion partners include bacterial β -galactosidase, trpE, Protein A, β -lactamase, alpha amylase, alcohol dehydrogenase, a FLAG fusion, and yeast alpha mating factor. See, e.g., Godowski, et al. (1988) Science 241:812-816.

20 The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the
25 strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation,
30 sulfonation, biotinylation, or the addition or removal of other moieties, particularly those which have molecular shapes similar to phosphate groups. In some embodiments, the modifications will be useful labeling reagents, or serve as purification targets, e.g.,
35 affinity tags as FLAG.

Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, for

example, in Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory. Techniques for synthesis of polypeptides are described, for example, in Merrifield (1963) J. Amer. Chem. Soc. 85:2149-2156; Merrifield (1986) Science 232: 341-347; and Atherton, et al. (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford; and chemical ligation, e.g., Dawson, et al. (1994) Science 266:776-779, a method of linking long synthetic peptides by a peptide bond.

This invention also contemplates the use of derivatives of these chemokines or receptors other than variations in amino acid sequence or glycosylation. Such derivatives may involve covalent or aggregative association with chemical moieties. These derivatives generally fall into the three classes: (1) salts, (2) side chain and terminal residue covalent modifications, and (3) adsorption complexes, for example with cell membranes. Such covalent or aggregative derivatives are useful as immunogens, as reagents in immunoassays, or in purification methods such as for affinity purification of ligands or other binding ligands. For example, a chemokine antigen can be immobilized by covalent bonding to a solid support such as cyanogen bromide-activated Sepharose, by methods which are well known in the art, or adsorbed onto polyolefin surfaces, with or without glutaraldehyde cross-linking, for use in the assay or purification of anti-chemokine antibodies or its receptor. These chemokines can also be labeled with a detectable group, for example radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates, or conjugated to a fluorescent moiety for use in diagnostic assays. Purification of chemokine may be effected by immobilized antibodies or receptor.

Other modifications may be introduced with the goal of modifying the therapeutic pharmacokinetics or pharmacodynamics of a target chemokine. For example, certain means to minimize the size of the entity may

improve its pharmacoaccessibility; other means to maximize size may affect pharmacodynamics.

A solubilized chemokine or appropriate fragment of this invention can be used as an immunogen for the
5 production of antisera or antibodies specific for the ligand or fragments thereof. The purified chemokines can be used to screen monoclonal antibodies or chemokine-binding fragments prepared by immunization with various forms of impure preparations containing the
10 protein. In particular, antibody equivalents include antigen binding fragments of natural antibodies, e.g., Fv, Fab, or F(ab)₂. Purified chemokines can also be used as a reagent to detect antibodies generated in response to the presence of elevated levels of the
15 protein or cell fragments containing the protein, both of which may be diagnostic of an abnormal or specific physiological or disease condition. Additionally, chemokine protein fragments, or their concatenates, may also serve as immunogens to produce antibodies of the
20 present invention, as described immediately below. For example, this invention contemplates antibodies raised against amino acid sequences shown in Tables 1 through 3, or proteins containing them. In particular, this
invention contemplates antibodies having binding
25 affinity to or being raised against specific fragments, e.g., those which are predicted to lie on the outside surfaces of protein tertiary structure. Similar concepts apply to antibodies specific for receptors of the invention.

30 The present invention contemplates the isolation of additional closely related species variants. Southern and Northern blot analysis should establish that similar genetic entities exist in other mammals, and establish the stringency of hybridization conditions to isolate
35 such. It is likely that these chemokines and receptors are widespread in species variants, e.g., rodents, lagomorphs, carnivores, artiodactyla, perissodactyla, and primates.

The invention also provides means to isolate a group of related chemokines displaying both distinctness and similarities in structure, expression, and function. Elucidation of many of the physiological effects of the proteins will be greatly accelerated by the isolation and characterization of distinct species variants of the ligands. In particular, the present invention provides useful probes for identifying additional homologous genetic entities in different species.

10 The isolated genes will allow transformation of cells lacking expression of a corresponding chemokine, e.g., either species types or cells which lack corresponding ligands and exhibit negative background activity. Expression of transformed genes will allow isolation of antigenically pure cell lines, with defined or single specie variants. This approach will allow for more sensitive detection and discrimination of the physiological effects of chemokine receptor proteins. Subcellular fragments, e.g., cytoplasts or membrane fragments, can be isolated and used.

20 Dissection of critical structural elements which effect the various differentiation functions provided by ligands is possible using standard techniques of modern molecular biology, particularly in comparing members of the related class. See, e.g., the homolog-scanning mutagenesis technique described in Cunningham, et al. (1989) Science 243:1339-1336; and approaches used in O'Dowd, et al. (1988) J. Biol. Chem. 263:15985-15992; and Lechleiter, et al. (1990) EMBO J. 9:4381-4390.

30 In addition, receptor binding segments can be substituted between species variants to determine what structural features are important in both receptor binding affinity and specificity, as well as signal transduction. An array of different chemokine variants will be used to screen for ligands exhibiting combined properties of interaction with different receptor species variants.

Intracellular functions would probably involve segments of the receptor which are normally accessible

to the cytosol. However, ligand internalization may occur under certain circumstances, and interaction between intracellular components and "extracellular" segments may occur. The specific segments of
5 interaction of a particular chemokine with other intracellular components may be identified by mutagenesis or direct biochemical means, e.g., cross-linking or affinity methods. Structural analysis by crystallographic or other physical methods will also be
10 applicable. Further investigation of the mechanism of signal transduction will include study of associated components which may be isolatable by affinity methods or by genetic means, e.g., complementation analysis of mutants.

15 Further study of the expression and control of the various chemokines will be pursued. The controlling elements associated with the proteins may exhibit differential developmental, tissue specific, or other expression patterns. Upstream or downstream genetic
20 regions, e.g., control elements, are of interest. Differential splicing of message may lead to membrane bound forms, soluble forms, and modified versions of ligand.

Structural studies of the proteins will lead to
25 design of new ligands, particularly analogs exhibiting agonist or antagonist properties on the receptor. This can be combined with previously described screening methods to isolate ligands exhibiting desired spectra of activities.

30 Expression in other cell types will often result in glycosylation differences in a particular chemokine. Various species variants may exhibit distinct functions based upon structural differences other than amino acid sequence. Differential modifications may be responsible
35 for differential function, and elucidation of the effects are now made possible.

Thus, the present invention provides important reagents related to a physiological chemokine-binding protein interaction. Although the foregoing description

has focused primarily upon the mouse and human
embodiments of the chemokines specifically described,
those of skill in the art will immediately recognize
that the invention provides other species counterparts,
5 e.g., rat and other mammalian species or allelic or
polymorphic variants.

V. Antibodies

Antibodies can be raised to these chemokines,
10 including species or polymorphic variants, and fragments
thereof, both in their naturally occurring forms and in
their recombinant forms. Additionally, antibodies can
be raised to chemokines in either their active forms or
in their inactive forms. Anti-idiotypic antibodies are
15 also contemplated.

Antibodies, including binding fragments and single
chain versions, against predetermined fragments of the
ligands can be raised by immunization of animals with
concatemers or conjugates of the fragments with
20 immunogenic proteins. Monoclonal antibodies are
prepared from cells secreting the desired antibody.
These antibodies can be screened for binding to normal
or defective chemokines, or screened for agonistic or
antagonistic activity, e.g., mediated through a receptor
25 for a chemokine. These monoclonal antibodies will
usually bind with at least a K_D of about 1 mM, more
usually at least about 300 μ M, typically at least about
10 μ M, more typically at least about 30 μ M, preferably
at least about 10 μ M, and more preferably at least about
30 3 μ M or better.

The antibodies, including antigen binding
fragments, of this invention can have significant
diagnostic or therapeutic value. They can be potent
antagonists that bind to ligand and inhibit binding to
35 receptor or inhibit the ability of a ligand to elicit a
biological response. They also can be useful as non-
neutralizing antibodies and can be coupled to toxins or
radionuclides so that when the antibody binds to ligand,
a cell expressing it, e.g., on its surface via receptor,

is killed. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker, and may effect drug targeting. Antibodies to receptors may be more easily
5 used to block ligand binding and signal transduction.

The antibodies of this invention can also be useful in diagnostic or reagent purification applications. As capture or non-neutralizing antibodies, they can be screened for ability to bind to the chemokines without
10 inhibiting receptor binding. As neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying chemokine or, indirectly, receptors, e.g., in immunoassays. They may be used as purification
15 reagents in immunoaffinity columns or as immunohistochemistry reagents.

Ligand fragments may be concatenated or joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens.
20 Short peptides will preferably be made as repeat structures to increase size. A ligand and its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See Microbiology,
25 Hoeber Medical Division, Harper and Row, 1969; Landsteiner (1962) Specificity of Serological Reactions, Dover Publications, New York, and Williams, et al. (1967) Methods in Immunology and Immunochemistry, Vol. 1, Academic Press, New York, for descriptions of methods
30 of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin fraction is isolated.

35 In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies may be found in, e.g., Stites, et al. (eds.) Basic and

Clinical Immunology (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH Press; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York; and particularly in Kohler and Milstein (1975) in Nature 256:495-497, which discusses one method of generating monoclonal antibodies. Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken, e.g., from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing in vitro. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance. Large amounts of antibody may be derived from ascites fluid from an animal.

Other suitable techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse, et al. (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281; and Ward, et al. (1989) Nature 341:544-546. The polypeptides and antibodies of the present invention may be used with or without modification, including chimeric or humanized antibodies. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors,

inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 5 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567; and Queen et al. (1989) Proc. Nat'l. Acad. Sci. 86:10029-10033.

The antibodies of this invention can also be used 10 for affinity chromatography in isolating the protein. Columns can be prepared where the antibodies are linked to a solid support, e.g., particles, such as agarose, Sephadex, or the like, where a cell lysate may be passed through the column, the column washed, followed by 15 increasing concentrations of a mild denaturant, whereby the purified chemokine protein will be released.

The antibodies may also be used to screen expression libraries for particular expression products. Usually the antibodies used in such a procedure will be 20 labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against these chemokine will also be useful to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various 25 immunological conditions related to expression of the respective antigens.

VI. Nucleic Acids

The described peptide sequences and the related 30 reagents are useful in isolating a DNA clone encoding these chemokines, e.g., from a natural source. Typically, it will be useful in isolating a gene from another individual, and similar procedures will be applied to isolate genes from other species, e.g., warm 35 blooded animals, such as birds and mammals. Cross hybridization will allow isolation of ligand from other species. A number of different approaches should be available to successfully isolate a suitable nucleic

acid clone. Similar concepts apply to the receptor embodiments.

The purified protein or defined peptides are useful for generating antibodies by standard methods, as
5 described above. Synthetic peptides or purified protein can be presented to an immune system to generate monoclonal or polyclonal antibodies. See, e.g., Coligan (1991) Current Protocols in Immunology Wiley/Greene; and Harlow and Lane (1989) Antibodies: A Laboratory Manual
10 Cold Spring Harbor Press. Alternatively, a chemokine receptor can be used as a specific binding reagent, and advantage can be taken of its specificity of binding, much like an antibody would be used. However, chemokine receptors are typically 7 transmembrane proteins, which
15 could be sensitive to appropriate interaction with lipid or membrane. The signal transduction typically is mediated through a G-protein.

For example, the specific binding composition could be used for screening of an expression library made from
20 a cell line which expresses a particular chemokine. The screening can be standard staining of surface expressed ligand, or by panning. Screening of intracellular expression can also be performed by various staining or immunofluorescence procedures. The binding compositions
25 could be used to affinity purify or sort out cells expressing the ligand.

The peptide segments can also be used to predict appropriate oligonucleotides to screen a library, e.g., to isolate species variants. The genetic code can be
30 used to select appropriate oligonucleotides useful as probes for screening. See, e.g., Tables 1 through 5. In combination with polymerase chain reaction (PCR) techniques, synthetic oligonucleotides will be useful in selecting correct clones from a library. Complementary
35 sequences will also be used as probes or primers. Based upon identification of the likely amino terminus, the third peptide should be particularly useful, e.g., coupled with anchored vector or poly-A complementary PCR techniques or with complementary DNA of other peptides.

This invention contemplates use of isolated DNA or fragments to encode a biologically active corresponding chemokine polypeptide. In addition, this invention covers isolated or recombinant DNA which encodes a
5 biologically active protein or polypeptide which is capable of hybridizing under appropriate conditions with the DNA sequences described herein. Said biologically active protein or polypeptide can be an intact ligand, or fragment, and have an amino acid sequence as
10 disclosed in Tables 1 through 3. Further, this invention covers the use of isolated or recombinant DNA, or fragments thereof, which encode proteins which are homologous to a chemokine or which was isolated using cDNA encoding a chemokine as a probe. The isolated DNA
15 can have the respective regulatory sequences in the 5' and 3' flanks, e.g., promoters, enhancers, poly-A addition signals, and others. Alternatively, promoters or other regulatory signals may be incorporated to be operably linked to natural genes in a cell.

20 An "isolated" nucleic acid is a nucleic acid, e.g., an RNA, DNA, or a mixed polymer, which is substantially separated from other components which naturally accompany a native sequence, e.g., ribosomes, polymerases, and flanking genomic sequences from the
25 originating species. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogs or analogs biologically synthesized by heterologous
30 systems. A substantially pure molecule includes isolated forms of the molecule.

An isolated nucleic acid will generally be a homogeneous composition of molecules, but will, in some embodiments, contain minor heterogeneity. This
35 heterogeneity is typically found at the polymer ends or portions not critical to a desired biological function or activity.

A "recombinant" nucleic acid is defined either by its method of production or its structure. In reference

to its method of production, e.g., a product made by a process, the process is use of recombinant nucleic acid techniques, e.g., involving human intervention in the nucleotide sequence, typically selection or production.

5 Alternatively, it can be a nucleic acid made by generating a sequence comprising fusion of two fragments which are not naturally contiguous to each other, but is meant to exclude products of nature, e.g., naturally occurring mutants. Thus, for example, products made by
10 transforming cells with any unnaturally occurring vector is encompassed, as are nucleic acids comprising sequence derived using any synthetic oligonucleotide process. Such is often done to replace a codon with a redundant codon encoding the same or a conservative amino acid,
15 while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the commonly
20 available natural forms. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features may be
25 incorporated by design. A similar concept is intended for a recombinant, e.g., fusion, polypeptide. Specifically included are synthetic nucleic acids which, by genetic code redundancy, encode polypeptides similar to fragments of these antigens, and fusions of sequences
30 from various different species variants.

A significant "fragment" in a nucleic acid context is a contiguous segment of at least about 17 nucleotides, generally at least about 20 nucleotides, more generally at least about 23 nucleotides, ordinarily
35 at least about 26 nucleotides, more ordinarily at least about 29 nucleotides, often at least about 32 nucleotides, more often at least about 35 nucleotides, typically at least about 38 nucleotides, more typically at least about 41 nucleotides, usually at least about 44

nucleotides, more usually at least about 47 nucleotides, preferably at least about 50 nucleotides, more preferably at least about 53 nucleotides, and in particularly preferred embodiments will be at least
5 about 56 or more nucleotides, e.g., 60, 65, 75, 85, 100, 120, 150, 200, 250, 300, 400, etc. Such fragments may have ends which begin and/or end at virtually all positions, e.g., beginning at nucleotides 1, 2, 3, etc., and ending at, e.g., 300, 299, 298, 287, etc., in all
10 combinations. Particularly interesting polynucleotides have ends corresponding to structural domain boundaries.

A DNA which codes for a particular chemokine protein or peptide will be very useful to identify genes, mRNA, and cDNA species which code for related or
15 homologous ligands, as well as DNAs which code for homologous proteins from different species. There are likely homologs in other species, including primates. Various chemokine proteins should be homologous and are encompassed herein. However, even proteins that have a
20 more distant evolutionary relationship to the ligand can readily be isolated under appropriate conditions using these sequences if they are sufficiently homologous. Primate chemokines are of particular interest.

This invention further covers recombinant DNA
25 molecules and fragments having a DNA sequence identical to or highly homologous to the isolated DNAs set forth herein. In particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication.
30 Alternatively, recombinant clones derived from the genomic sequences, e.g., containing introns, will be useful for transgenic studies, including, e.g., transgenic cells and organisms, and for gene therapy. See, e.g., Goodnow (1992) "Transgenic Animals" in Roitt
35 (ed.) Encyclopedia of Immunology Academic Press, San Diego, pp. 1502-1504; Travis (1992) Science 256:1392-1394; Kuhn, et al. (1991) Science 254:707-710; Capecchi (1989) Science 244:1288; Robertson (1987) (ed.) Teratocarcinomas and Embryonic Stem Cells: A Practical

Approach IRL Press, Oxford; and Rosenberg (1992) J. Clinical Oncology 10:180-199.

Homologous nucleic acid sequences, when compared, exhibit significant similarity, or identity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon hybridization conditions. The hybridization conditions are described in greater detail below.

Substantial homology in the nucleic acid sequence comparison context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 50% of the nucleotides, generally at least about 56%, more generally at least about 59%, ordinarily at least about 62%, more ordinarily at least about 65%, often at least about 68%, more often at least about 71%, typically at least about 74%, more typically at least about 77%, usually at least about 80%, more usually at least about 85%, preferably at least about 90%, more preferably at least about 95 to 98% or more, and in particular embodiments, as high at about 99% or more of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, typically using a sequence derived from Tables 1 through 5. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 30 nucleotides, preferably at least about 65% over a stretch of at least about 25 nucleotides, more preferably at least about 75%, and most preferably at least about 90% over about 20 nucleotides. See, Kanehisa (1984) Nuc. Acids Res. 12:203-213. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at

least about 28 nucleotides, more typically at least about 40 nucleotides, preferably at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides.

5 Stringent conditions, in referring to homology in the hybridization context, will be stringent combined conditions of salt, temperature, organic solvents, and other parameters, typically those controlled in hybridization reactions. Stringent temperature
10 conditions will usually include temperatures in excess of about 30° C, more usually in excess of about 37° C, typically in excess of about 45° C, more typically in excess of about 55° C, preferably in excess of about 65° C, and more preferably in excess of about 70° C.
15 Stringent salt conditions will ordinarily be less than about 1000 mM, usually less than about 500 mM, more usually less than about 400 mM, typically less than about 300 mM, preferably less than about 200 mM, and more preferably less than about 150 mM. However, the
20 combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur and Davidson (1968) J. Mol. Biol. 31:349-370.

Corresponding chemokines from other mammalian species can be cloned and isolated by cross-species
25 hybridization of closely related species. Alternatively, sequences from a data base may be recognized as having similarity. Homology may be relatively low between distantly related species, and thus hybridization of relatively closely related species
30 is advisable. Alternatively, preparation of an antibody preparation which exhibits less species specificity may be useful in expression cloning approaches. PCR approaches using segments of conserved sequences will also be used.

35

VII. Making chemokines, receptors; Mimetics

DNA which encodes each respective chemokine or fragments thereof can be obtained by chemical synthesis, screening cDNA libraries, or by screening genomic

libraries prepared from a wide variety of cell lines or tissue samples.

This DNA can be expressed in a wide variety of host cells for the synthesis of a full-length ligand or
5 fragments which can in turn, for example, be used to generate polyclonal or monoclonal antibodies; for binding studies; for construction and expression of modified molecules; and for structure/function studies. Each antigen or its fragments can be expressed in host
10 cells that are transformed or transfected with appropriate expression vectors. These molecules can be substantially purified to be free of protein or cellular contaminants, other than those derived from the recombinant host, and therefore are particularly useful
15 in pharmaceutical compositions when combined with a pharmaceutically acceptable carrier and/or diluent. The antigen, or portions thereof, may be expressed as fusions with other proteins.

Expression vectors are typically self-replicating
20 DNA or RNA constructs containing the desired antigen gene or its fragments, usually operably linked to suitable genetic control elements that are recognized in a suitable host cell. These control elements are capable of effecting expression within a suitable host.
25 The specific type of control elements necessary to effect expression will depend upon the eventual host cell used. Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system, and typically
30 include a transcriptional promoter, an optional operator to control the onset of transcription, transcription enhancers to elevate the level of mRNA expression, a sequence that encodes a suitable ribosome binding site, and sequences that terminate transcription and
35 translation. Expression vectors also usually contain an origin of replication that allows the vector to replicate independently of the host cell.

The vectors of this invention contain DNA which encode embodiments of a chemokine, receptor, or a

fragment thereof, typically encoding a biologically active polypeptide. The DNA can be under the control of a viral promoter and can encode a selection marker. This invention further contemplates use of such

5 expression vectors which are capable of expressing eukaryotic cDNA coding for each chemokine in a prokaryotic or eukaryotic host, where the vector is compatible with the host and where the eukaryotic cDNA coding for the ligand is inserted into the vector such

10 that growth of the host containing the vector expresses the cDNA in question. Usually, expression vectors are designed for stable replication in their host cells or for amplification to greatly increase the total number of copies of the desirable gene per cell. It is not

15 always necessary to require that an expression vector replicate in a host cell, e.g., it is possible to effect transient expression of the ligand or its fragments in various hosts using vectors that do not contain a replication origin that is recognized by the host cell.

20 It is also possible to use vectors that cause integration of a chemokine gene or its fragments into the host DNA by recombination, or to integrate a promoter which controls expression of an endogenous gene.

25 Vectors, as used herein, comprise plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles which enable the integration of DNA fragments into the genome of the host. Expression vectors are specialized vectors which contain genetic

30 control elements that effect expression of operably linked genes. Plasmids are the most commonly used form of vector but all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g.,

35 Pouwels, et al. (1985 and Supplements) Cloning Vectors: A Laboratory Manual, Elsevier, N.Y., and Rodriquez, et al. (1988) (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworth, Boston, MA.

Transformed cells include cells, preferably mammalian, that have been transformed or transfected with a chemokine gene containing vector constructed using recombinant DNA techniques. Transformed host
5 cells usually express the ligand or its fragments, but for purposes of cloning, amplifying, and manipulating its DNA, do not need to express the protein. This invention further contemplates culturing transformed cells in a nutrient medium, thus permitting the protein
10 to accumulate in the culture. The protein can be recovered, either from the culture or from the culture medium.

For purposes of this invention, DNA sequences are operably linked when they are functionally related to
15 each other. For example, DNA for a presequence or secretory leader is operably linked to a polypeptide if it is expressed as a preprotein or participates in directing the polypeptide to the cell membrane or in secretion of the polypeptide. A promoter is operably
20 linked to a coding sequence if it controls the transcription of the polypeptide; a ribosome binding site is operably linked to a coding sequence if it is positioned to permit translation. Usually, operably linked means contiguous and in reading frame, however,
25 certain genetic elements such as repressor genes are not contiguously linked but still bind to operator sequences that in turn control expression.

Suitable host cells include prokaryotes, lower eukaryotes, and higher eukaryotes. Prokaryotes include
30 both gram negative and gram positive organisms, e.g., *E. coli* and *B. subtilis*. Lower eukaryotes include yeasts, e.g., *S. cerevisiae* and *Pichia*, and species of the genus *Dictyostelium*. Higher eukaryotes include established tissue culture cell lines from animal cells, both of
35 non-mammalian origin, e.g., insect cells, and birds, and of mammalian origin, e.g., human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. As used herein, *E. coli* and its vectors will be used generically

to include equivalent vectors used in other prokaryotes. A representative vector for amplifying DNA is pBR322 or many of its derivatives. Vectors that can be used to express these chemokines or their fragments include, but
5 are not limited to, such vectors as those containing the lac promoter (pUC-series); trp promoter (pBR322-trp); Ipp promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as ptac (pDR540). See Brosius, et al. (1988) "Expression Vectors Employing
10 Lambda-, trp-, lac-, and Ipp-derived Promoters", in Rodriguez and Denhardt (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Buttersworth, Boston, Chapter 10, pp. 205-236.

Lower eukaryotes, e.g., yeasts and Dictyostelium,
15 may be transformed with chemokine sequence containing vectors. For purposes of this invention, the most common lower eukaryotic host is the baker's yeast, *Saccharomyces cerevisiae*. It will be used to generically represent lower eukaryotes although a number
20 of other strains and species are also available. Yeast vectors typically consist of a replication origin (unless of the integrating type), a selection gene, a promoter, DNA encoding the desired protein or its fragments, and sequences for translation termination,
25 polyadenylation, and transcription termination. Suitable expression vectors for yeast include such constitutive promoters as 3-phosphoglycerate kinase and various other glycolytic enzyme gene promoters or such inducible promoters as the alcohol dehydrogenase 2
30 promoter or metallothioneine promoter. Suitable vectors include derivatives of the following types: self-replicating low copy number (such as the YRp-series), self-replicating high copy number (such as the YEp-series); integrating types (such as the YIp-series), or
35 mini-chromosomes (such as the YCp-series).

Higher eukaryotic tissue culture cells are the preferred host cells for expression of the functionally active chemokine protein. In principle, most any higher eukaryotic tissue culture cell line is workable, e.g.,

insect baculovirus expression systems, whether from an invertebrate or vertebrate source. However, mammalian cells are preferred, in that the processing, both cotranslationally and posttranslationally.

5 Transformation or transfection and propagation of such cells has become a routine procedure. Examples of useful cell lines include HeLa cells, Chinese hamster ovary (CHO) cell lines, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey
10 (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors
15 also usually contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, e.g., from such sources as from adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus.
20 Representative examples of suitable expression vectors include pCDNA1; pCD, see Okayama, et al. (1985) Mol. Cell Biol. 5:1136-1142; pMC1neo Poly-A, see Thomas, et al. (1987) Cell 51:503-512; and a baculovirus vector such as pAC 373 or pAC 610.

25 It will often be desired to express a chemokine polypeptide in a system which provides a specific or defined glycosylation pattern. In this case, the usual pattern will be that provided naturally by the expression system. However, the pattern will be
30 modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, a chemokine gene may be co-transformed with one or more genes encoding mammalian or
35 other glycosylating enzymes. Using this approach, certain mammalian glycosylation patterns will be achievable or approximated in prokaryote or other cells.

A chemokine, or a fragment thereof, may be engineered to be phosphatidyl inositol (PI) linked to a

cell membrane, but can be removed from membranes by treatment with a phosphatidyl inositol cleaving enzyme, e.g., phosphatidyl inositol phospholipase-C. This releases the antigen in a biologically active form, and allows purification by standard procedures of protein chemistry. See, e.g., Low (1989) Biochim. Biophys. Acta 988:427-454; Tse, et al. (1985) Science 230:1003-1008; and Brunner, et al. (1991) J. Cell Biol. 114:1275-1283.

Now that these chemokines have been characterized, fragments or derivatives thereof can be prepared by conventional processes for synthesizing peptides. These include processes such as are described in Stewart and Young (1984) Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL; Bodanszky and Bodanszky (1984) The Practice of Peptide Synthesis, Springer-Verlag, New York; and Bodanszky (1984) The Principles of Peptide Synthesis, Springer-Verlag, New York. For example, an azide process, an acid chloride process, an acid anhydride process, a mixed anhydride process, an active ester process (for example, p-nitrophenyl ester, N-hydroxysuccinimide ester, or cyanomethyl ester), a carbodiimidazole process, an oxidative-reductive process, or a dicyclohexylcarbodiimide (DCCD)/additive process can be used. Solid phase and solution phase syntheses are both applicable to the foregoing processes.

These chemokines, fragments, or derivatives are suitably prepared in accordance with the above processes as typically employed in peptide synthesis, generally either by a so-called stepwise process which comprises condensing an amino acid to the terminal amino acid, one by one in sequence, or by coupling peptide fragments to the terminal amino acid. Amino groups that are not being used in the coupling reaction are typically protected to prevent coupling at an incorrect location.

If a solid phase synthesis is adopted, the C-terminal amino acid is typically bound to an insoluble carrier or support through its carboxyl group. The insoluble carrier is not particularly limited as long as

it has a binding capability to a reactive carboxyl group. Examples of such insoluble carriers include halomethyl resins, such as chloromethyl resin or bromomethyl resin, hydroxymethyl resins, phenol resins, 5 tert-alkyloxycarbonyl-hydrazidated resins, and the like.

An amino group-protected amino acid is bound in sequence through condensation of its activated carboxyl group and the reactive amino group of the previously formed peptide or chain, to synthesize the peptide step 10 by step. After synthesizing the complete sequence, the peptide is split off from the insoluble carrier to produce the peptide. This solid-phase approach is generally described by Merrifield, et al. (1963) in J. Am. Chem. Soc. 85:2149-2156.

15 The prepared ligand and fragments thereof can be isolated and purified from the reaction mixture by means of peptide separation, e.g., by extraction, precipitation, electrophoresis and various forms of chromatography, and the like. The various chemokines of 20 this invention can be obtained in varying degrees of purity depending upon its desired use. Purification can be accomplished by use of the protein purification techniques disclosed herein or by the use of the antibodies herein described, e.g., in immunoabsorbant 25 affinity chromatography. This immunoabsorbant affinity chromatography is carried out by first linking the antibodies to a solid support and then contacting the linked antibodies with solubilized lysates of appropriate source cells, lysates of other cells 30 expressing the ligand, or lysates or supernatants of cells producing the desired chemokine as a result of DNA techniques, see below.

VIII. Uses

35 The present invention provides reagents which will find use in diagnostic applications as described elsewhere herein, e.g., in the general description for developmental abnormalities, or below in the description of kits for diagnosis.

This invention also provides reagents with significant therapeutic value. These chemokines (naturally occurring or recombinant), fragments thereof and antibodies thereto, along with compounds identified
5 as having binding affinity to them, should be useful in the treatment of conditions associated with abnormal physiology or development, including inflammatory conditions, including asthma. In particular, modulation of trafficking of leukocytes is one likely biological
10 activity, but a wider tissue distribution might suggest broader biological activity, including, e.g., antiviral effects. Abnormal proliferation, regeneration, degeneration, and atrophy may be modulated by appropriate therapeutic treatment using the compositions
15 provided herein. For example, a disease or disorder associated with abnormal expression or abnormal signaling by a chemokine should be a likely target for an agonist or antagonist of the ligand.

Various abnormal physiological or developmental
20 conditions are known in cell types shown to possess the chemokine mRNAs by Northern blot analysis. See Berkow (ed.) The Merck Manual of Diagnosis and Therapy, Merck & Co., Rahway, N.J.; and Thorn, et al. Harrison's Principles of Internal Medicine, McGraw-Hill, N.Y.
25 Developmental or functional abnormalities, e.g., of the immune system, cause significant medical abnormalities and conditions which may be susceptible to prevention or treatment using compositions provided herein.

Chemokine antibodies, including recombinant forms,
30 can be purified and then administered to a patient. These reagents can be combined for therapeutic use with additional active or inert ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g., immunogenic adjuvants, along with
35 physiologically innocuous stabilizers and excipients. These combinations can be sterile filtered and placed into dosage forms as by lyophilization in dosage vials or storage in stabilized aqueous preparations. This invention also contemplates use of antibodies or binding

fragments thereof, including forms which are not complement binding. Moreover, modifications to the antibody molecules or antigen binding fragments thereof, may be adopted which affect the pharmacokinetics or pharmacodynamics of the therapeutic entity.

Drug screening using antibodies or receptor or fragments thereof can be performed to identify compounds having binding affinity to each chemokine or receptor, including isolation of associated components.

Subsequent biological assays can then be utilized to determine if the compound has intrinsic stimulating activity and is therefore a blocker or antagonist in that it blocks the activity of the ligand. Likewise, a compound having intrinsic stimulating activity can activate the receptor and is thus an agonist in that it simulates the activity of a chemokine. This invention further contemplates the therapeutic use of antibodies to these chemokines as antagonists. This approach should be particularly useful with other chemokine species variants.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy in various populations, including racial subgroups, age, gender, etc. Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman, et al. (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn.. Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular

administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers typically include water, saline, buffers, and other compounds described, e.g., in the Merck Index, Merck & Co., Rahway, New

5 Jersey. Dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μ M concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM
10 (femtomolar), with an appropriate carrier. Slow release formulations, or a slow release apparatus will often be utilized for continuous administration.

A chemokine, fragments thereof, or antibodies to it or its fragments, antagonists, and agonists, may be
15 administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to carrier proteins such as ovalbumin or serum albumin prior to their administration. Therapeutic formulations may be
20 administered in any conventional dosage formulation. While it is possible for the active ingredient to be administered alone, it is often preferable to present it as a pharmaceutical formulation. Formulations typically comprise at least one active ingredient, as defined
25 above, together with one or more acceptable carriers thereof. Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Carriers may improve storage life,
30 stability, etc. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form and may be
35 prepared by any methods well known in the art of pharmacy. See, e.g., Gilman, et al. (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack

Publishing Co., Easton, Penn.; Avis, et al. (eds.)
(1993) Pharmaceutical Dosage Forms: Parenteral Medications Dekker, New York; Lieberman, et al. (eds.)
(1990) Pharmaceutical Dosage Forms: Tablets Dekker, New
5 York; and Lieberman, et al. (eds.) (1990) Pharmaceutical Dosage Forms: Disperse Systems Dekker, New York. The therapy of this invention may be combined with or used in association with other therapeutic agents.

Both the naturally occurring and the recombinant
10 forms of the chemokines of this invention are particularly useful in kits and assay methods which are capable of screening compounds for binding activity to the proteins. Several methods of automating assays have been developed in recent years so as to permit screening
15 of tens of thousands of compounds in a short period. See, e.g., Fodor, et al. (1991) Science 251:767-773, which describes means for testing of binding affinity by a plurality of defined polymers synthesized on a solid substrate. The development of suitable assays can be
20 greatly facilitated by the availability of large amounts of purified, soluble chemokine as provided by this invention.

For example, antagonists can normally be found once the ligand has been structurally defined. Testing of
25 potential ligand analogs is now possible upon the development of highly automated assay methods using physiologically responsive cells. In particular, new agonists and antagonists will be discovered by using screening techniques described herein.

30 Viable cells could also be used to screen for the effects of drugs on respective chemokine mediated functions, e.g., second messenger levels, i.e., Ca^{++} ; inositol phosphate pool changes (see, e.g., Berridge (1993) Nature 361:315-325 or Billah and Anthes (1990)
35 Biochem. J. 269:281-291); cellular morphology modification responses; phosphoinositide lipid turnover; an antiviral response. and others. Some detection methods allow for elimination of a separation step, e.g., a proximity sensitive detection system. Calcium

sensitive dyes will be useful for detecting Ca^{++} levels, with a fluorimeter or a fluorescence cell sorting apparatus.

Rational drug design may also be based upon structural studies of the molecular shapes of the chemokines and other effectors or analogs. Effectors may be other proteins which mediate other functions in response to ligand binding, or other proteins which normally interact with the receptor. One means for determining which sites interact with specific other proteins is a physical structure determination, e.g., x-ray crystallography or 2 dimensional NMR techniques. These will provide guidance as to which amino acid residues form molecular contact regions. For a detailed description of protein structural determination, see, e.g., Blundell and Johnson (1976) Protein Crystallography, Academic Press, New York.

Purified chemokine can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to these ligands can be used as capture antibodies to immobilize the respective ligand on the solid phase.

Similar concepts also apply to the chemokine receptor embodiments of the invention.

IX. Kits

This invention also contemplates use of chemokine proteins, fragments thereof, peptides, and their fusion products in a variety of diagnostic kits and methods for detecting the presence of ligand, antibodies, or chemokine receptors. Typically the kit will have a compartment containing either a defined chemokine peptide or gene segment or a reagent which recognizes one or the other, e.g., antibodies.

A kit for determining the binding affinity of a test compound to a chemokine would typically comprise a test compound; a labeled compound, for example an antibody having known binding affinity for the ligand; a source of chemokine (naturally occurring or

recombinant); and a means for separating bound from free labeled compound, such as a solid phase for immobilizing the ligand. Once compounds are screened, those having suitable binding affinity to the ligand can be evaluated in suitable biological assays, as are well known in the art, to determine whether they act as agonists or antagonists to the receptor. The availability of recombinant chemokine polypeptides also provide well defined standards for calibrating such assays or as positive control samples.

A preferred kit for determining the concentration of, for example, a chemokine in a sample would typically comprise a labeled compound, e.g., antibody, having known binding affinity for the ligand, a source of ligand (naturally occurring or recombinant) and a means for separating the bound from free labeled compound, for example, a solid phase for immobilizing the chemokine. Compartments containing reagents, and instructions for use or disposal, will normally be provided.

Antibodies, including antigen binding fragments, specific for the chemokine or ligand fragments are useful in diagnostic applications to detect the presence of elevated levels of chemokine and/or its fragments. Such diagnostic assays can employ lysates, live cells, fixed cells, immunofluorescence, cell cultures, body fluids, and further can involve the detection of antigens related to the ligand in serum, or the like. Diagnostic assays may be homogeneous (without a separation step between free reagent and antigen-ligand complex) or heterogeneous (with a separation step). Various commercial assays exist, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), enzyme-multiplied immunoassay technique (EMIT), substrate-labeled fluorescent immunoassay (SLFIA), and the like. For example, unlabeled antibodies can be employed by using a second antibody which is labeled and which recognizes the antibody to a chemokine or to a particular fragment thereof. Similar assays have also

been extensively discussed in the literature. See, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH.

Anti-idiotypic antibodies may have similar uses to
5 diagnose presence of antibodies against a chemokine, as
such may be diagnostic of various abnormal states. For
example, overproduction of a chemokine may result in
production of various immunological reactions which may
be diagnostic of abnormal physiological states,
10 particularly in various inflammatory or asthma
conditions.

Frequently, the reagents for diagnostic assays are
supplied in kits, so as to optimize the sensitivity of
the assay. For the subject invention, depending upon
15 the nature of the assay, the protocol, and the label,
either labeled or unlabeled antibody or labeled
chemokine is provided. This is usually in conjunction
with other additives, such as buffers, stabilizers,
materials necessary for signal production such as
20 substrates for enzymes, and the like. Preferably, the
kit will also contain instructions for proper use and
disposal of the contents after use. Typically the kit
has compartments for each useful reagent. Desirably,
the reagents are provided as a dry lyophilized powder,
25 where the reagents may be reconstituted in an aqueous
medium providing appropriate concentrations of reagents
for performing the assay.

The aforementioned constituents of the drug
screening and the diagnostic assays may be used without
30 modification or may be modified in a variety of ways.
For example, labeling may be achieved by covalently or
non-covalently joining a moiety which directly or
indirectly provides a detectable signal. In any of
these assays, the ligand, test compound, chemokine, or
35 antibodies thereto can be labeled either directly or
indirectly. Possibilities for direct labeling include
label groups: radiolabels such as ^{125}I , enzymes (U.S.
Pat. No. 3,645,090) such as peroxidase and alkaline
phosphatase, and fluorescent labels (U.S. Pat. No.

3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Possibilities for indirect labeling include biotinylation of one constituent
5 followed by binding to avidin coupled to one of the above label groups.

There are also numerous methods of separating bound from the free ligand, or alternatively bound from free test compound. The chemokine can be immobilized on
10 various matrixes followed by washing. Suitable matrixes include plastic such as an ELISA plate, filters, and beads. Methods of immobilizing the chemokine to a matrix include, without limitation, direct adhesion to plastic, use of a capture antibody, chemical coupling,
15 and biotin-avidin. The last step in this approach involves the precipitation of ligand/antibody complex by any of several methods including those utilizing, e.g., an organic solvent such as polyethylene glycol or a salt such as ammonium sulfate. Other suitable separation
20 techniques include, without limitation, the fluorescein antibody magnetizable particle method described in Rattle, et al. (1984) Clin. Chem. 30:1457-1461, and the double antibody magnetic particle separation as described in U.S. Pat. No. 4,659,678.

25 Methods for linking proteins or their fragments to the various labels have been extensively reported in the literature and do not require detailed discussion here. Many of the techniques involve the use of activated carboxyl groups either through the use of carbodiimide
30 or active esters to form peptide bonds, the formation of thioethers by reaction of a mercapto group with an activated halogen such as chloroacetyl, or an activated olefin such as maleimide, for linkage, or the like. Fusion proteins will also find use in these
35 applications.

Another diagnostic aspect of this invention involves use of oligonucleotide or polynucleotide sequences taken from the sequence of a chemokine. These sequences can be used as probes for detecting levels of

the ligand message in samples from patients suspected of having an abnormal condition, e.g., an inflammatory or developmental problem. The preparation of both RNA and DNA nucleotide sequences, the labeling of the sequences, and the preferred size of the sequences has received ample description and discussion in the literature. Normally an oligonucleotide probe should have at least about 14 nucleotides, usually at least about 18 nucleotides, and the polynucleotide probes may be up to several kilobases. Various labels may be employed, most commonly radionuclides, particularly ^{32}P . However, other techniques may also be employed, such as using biotin modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed which can recognize specific duplexes, including DNA duplexes, RNA duplexes, DNA-RNA hybrid duplexes, or DNA-protein duplexes. The antibodies in turn may be labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. The use of probes to the novel anti-sense RNA may be carried out in any conventional techniques such as nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid released translation (HRT), and hybrid arrested translation (HART). This also includes amplification techniques such as polymerase chain reaction (PCR).

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of multiple indications used as markers. Thus, kits may test for combinations of markers. See, e.g., Viallet, et al. (1989) Progress in Growth Factor Res. 1:89-97.

X. Receptor

Having isolated a ligand binding partner of a specific interaction, methods exist for isolating the counter-partner. See, Gearing, et al EMBO J. 8:3667-4676 or McMahan, et al. (1991) EMBO J. 10:2821-2832. For example, means to label a chemokine without interfering with the binding to its receptor can be determined. For example, an affinity label can be fused to either the amino- or carboxy-terminus of the ligand. An expression library can be screened for specific binding of chemokine, e.g., by cell sorting, or other screening to detect subpopulations which express such a binding component. See, e.g., Ho, et al. (1993) Proc. Nat'l Acad. Sci. 90:11267-11271. Alternatively, a panning method may be used. See, e.g., Seed and Aruffo (1987) Proc. Nat'l. Acad. Sci. 84:3365-3369.

Protein cross-linking techniques with label can be applied to a isolate binding partners of a chemokine. This would allow identification of protein which specifically interacts with a chemokine, e.g., in a ligand-receptor like manner.

In various embodiments, new receptors designated DC CR and M/DC CR were isolated. The sequences of the human constructs and product are provided in Tables 4 and 5. Similar means for making variants and fragments, at the nucleotide level or at the protein level, and making antibodies will be available as described above, directed primarily to the chemokine embodiments. Many similar or related uses to the ligands will be applied to the receptors, as specific binding reagents. In particular, methods will be applied to screening for specific ligands for each receptor. Many uses, including kits, will also be available through analogous techniques.

The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the invention to specific embodiments.

EXAMPLES

I. General Methods

Some of the standard methods are described or
5 referenced, e.g., in Maniatis, et al. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual, (2d ed.), vols 1-3, CSH Press, NY; Ausubel, et al., Biology,
10 Greene Publishing Associates, Brooklyn, NY; or Ausubel, et al. (1987 and Supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York; Innis, et al. (eds.) (1990) PCR Protocols: A Guide to Methods and Applications Academic Press, N.Y. Methods for protein
15 purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel, et al. (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification" in
20 Methods in Enzymology, vol. 182, and other volumes in this series; and manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond, CA. Combination with recombinant techniques allow fusion to appropriate
25 segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See, e.g., Hochuli (1989) Chemische Industrie 12:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12:87-98, Plenum
30 Press, N.Y.; and Crowe, et al. (1992) QIAexpress: The High Level Expression & Protein Purification System QUIAGEN, Inc., Chatsworth, CA.

FACS analyses are described in Melamed, et al.
35 (1990) Flow Cytometry and Sorting Wiley-Liss, Inc., New York, NY; Shapiro (1988) Practical Flow Cytometry Liss, New York, NY; and Robinson, et al. (1993) Handbook of Flow Cytometry Methods Wiley-Liss, New York, NY.

II. Isolation and characterization of chemokine cDNAs

A. TECK

The TECK was isolated from a cDNA library made from thymus cells from a RAG-1 "knockout" mouse. See,
5 Mombaerts, et al. (1992) Cell 68:869-877. Individual
cDNA clones were sequenced using standard methods, e.g.,
the Taq DyeDeoxy Terminator Cycle Sequencing kit
(Applied Biosystems, Foster City, CA), and the TECK
sequence was identified and further characterized.
10 Computer analyses with other C-C chemokine family
members revealed significant homology at the amino acid
levels with other chemokines. The nucleotide sequence
for mouse is provided in Table 1, encoding a polypeptide
of about 144 amino acids. The signal sequence should
15 run from 1 (met) to about 23 (ala), and removal of the
signal sequence should provide one natural mature
sequence beginning at 24 (gln). Additional processing
may occur in a physiological system.

The sequence is notable in having a longer carboxy-
20 terminal tail than most other CC chemokines. TECK
exhibits one glycosylation site, and several AAMP, PKC,
and CK2 phosphorylation sites.

B. MIP-3 α

The MIP-3 α was isolated from a cDNA library made
25 from human monocytes activated with LPS and IFN- γ
Individual cDNA clones were sequenced using standard
methods, and the MIP-3 α sequence was identified and
further characterized. The nucleotide sequence is
provided in Table 2, encoding a polypeptide of at least
30 about 89 amino acids. The signal sequence should run
from about 1 (met) to 21 (cys), and removal of the
signal sequence should provide one natural sequence
beginning with gly. Additional processing may occur in
a physiological system.

C. MIP-3 β

The MIP-3 β was isolated from a cDNA library made from human fetal lung cells. Individual cDNA clones are sequenced using standard methods, and the MIP-3 α sequence was identified and further characterized. The nucleotide sequence is provided in Table 3, encoding a polypeptide of about 98 amino acids. The signal sequence should run from about 1 (met) to about 21 (ser), and removal of the signal sequence should provide one mature natural sequence beginning from gly. Additional processing may occur in a physiological system.

This chemokine has been paired with a receptor designated Eb11. See Yoshida, et al. (1997) J. Biol. Chem. 13803-13809.

D. Dendritic Cell Receptor for chemokine; DC CR

The DC CR was isolated from RNA made from dendritic cells isolated from CD34⁺ cord blood cells, isolated by standard procedure. It was also isolated from eosinophils using degenerate PCR primers of the TM2 and TM7 segments, which are often conserved among chemokine receptors. These eosinophils were isolated by taking PBLs, depletion of red blood cells by lysis, and negative selection of CD16 to remove neutrophils.

Sequencing of the PCR fragments indicated a potential novel receptor, and the fragment was used to isolate a full length clone by hybridization. Clone isolates were sequenced using standard methods, and the DC CR sequence was identified and further characterized. The nucleotide sequence is provided in Table 4, encoding a polypeptide of about 365 amino acids. The transmembrane segments, determined by homology to the IL-8 B receptor, are about: TM1 from 39 (leu) to 64 (phe); TM2 from 76 (leu) to 96 (ser); TM3 from 111 (leu) to 132 (met); TM4 from 151 (thr) to 176 (phe); TM5 from 207 (gly) to 229 (val); TM6 from 246 (val) to 270 (ala); and TM7 from 291 (val) to 319 (leu). The amino terminal segment is probably an extracellular segment, and the others would be between TM2 and TM3; and TM4 and TM5;

and TM6 and TM7. The intracellular segments should then run between TM1 and TM2; TM3 and TM4, TM5 and TM6, and the carboxy terminus from the end of TM7. Additional processing may occur in a physiological system.

5 The implication of chemokine receptors in retroviral infection suggest that the receptor may be critical for infection. Antibodies which block infection may be routinely screened, and developed for therapeutic uses.

10 E. Monocyte/Dendritic Cell Receptor for chemokine;
M/DC CR

 The M/DC CR was isolated from a cDNA library made from human monocyte cells cultured for 2.5 to 4 h in medium containing IFN- γ (10 ng/ml), LPS (1 μ g/ml), anti-IL-4 monoclonal antibody (5 μ g/ml), and anti-IL-10 monoclonal antibody (5 μ g/ml). Individual cDNA clones were sequenced using standard methods, and the M/DC CR sequence was identified and further characterized. The nucleotide sequence is provided in Table 5, encoding a polypeptide of about 356 amino acids. The transmembrane segments, should be about as follows: TM1 from 52 (leu) to 76 (val); TM2 from 86 (asn) to 107 (ala); TM3 from 117 (ile) to 138 (val); TM4 from 157 (val) to 182 (tyr); TM5 from 211 (phe) to 233 (val); TM6 from 251 (leu) to 275 (phe); and TM7 from 296 (ile) to 315 (leu). As for the DC CR, the amino terminal segment is probably an extracellular segment, and the others would be between TM2 and TM3; and TM4 and TM5; and TM6 and TM7. The intracellular segments should then run between TM1 and TM2; TM3 and TM4, TM5 and TM6, and the carboxy terminus from the end of TM7.

III. Preparation of antibodies

 Many standard methods are available for preparation of antibodies. For example, synthetic peptides may be prepared to be used as antigen, administered to an appropriate animal, and either polyclonal or monoclonal antibodies prepared. Short peptides, e.g., less than about 10 amino acids may be repeated, while longer

peptides may be used alone or conjugated to a carrier. For example, with the M/DC CR, animals were immunized with peptides corresponding to amino acid sequences from 18-44 (starting with LAP and ending with KYD; a fragment
5 towards the amino terminus) and from 183-204 (starting with KPQ and ending with PAD; corresponding to an extracellular loop), see SEQ ID NO: 13. Highest specificity will result when the polypeptides are selected from portions which are most unique, e.g., not
10 form conserved sequence regions. The animals may be used to collect antiserum, or may be used to generate monoclonal antibodies.

Antiserum was determined useful for ELISA, and will be evaluated for utility as immunoprecipitation or
15 Western blot analysis. Monoclonal antibodies will also be evaluated for those same uses.

The antibodies provided will be useful as immunoaffinity reagents, as detection reagents, for immunohistochemistry, and as therapeutic reagents.
20

IV. Assays for chemotactic activity of chemokines.

Chemokine proteins are produced, e.g., in COS cells transfected with a plasmid carrying the chemokine cDNA by electroporation. See, Hara, et al. (1992) EMBO J.
25 10:1875-1884. Physical analytical methods may be applied, e.g., CD analysis, to compare tertiary structure to other chemokines to evaluate whether the protein has likely folded into an active conformation. After transfection, a culture supernatant is collected
30 and subjected to bioassays. A mock control, e.g., a plasmid carrying the luciferase cDNA, is used. See, de Wet, et al. (1987) Mol. Cell. Biol. 7:725-757. A positive control, e.g., recombinant murine MIP-1 α from R&D Systems (Minneapolis, MN), is typically used.
35 Likewise, antibodies may be used to block the biological activities, e.g., as a control.

Lymphocyte migration assays are performed as previously described, e.g., in Bacon, et al. (1988) Br. J. Pharmacol. 95:966-974. Murine Th2 T cell clones,

CDC-25 (see Tony, et al. (1985) J. Exp. Med. 161:223-241) and HDK-1 (see Cherwinski, et al. (1987) J. Exp. Med. 166:1229-1244), made available from R. Coffman and A. O'Garra (DNAX, Palo Alto, CA), respectively, are used
5 as controls.

Ca²⁺ flux upon chemokine stimulation is measured according to the published procedure described in Bacon, et al. (1995) J. Immunol. 154:3654-3666.

Maximal numbers of migrating cells in response to
10 MIP-1 α typically occur at a concentration of 10⁻⁸ M, in agreement with original reports for CD4⁺ populations of human T cells. See Schall (1993) J. Exp. Med. 177:1821-1826. A dose-response curve is determined, preferably giving a characteristic bell shaped dose-
15 response curve.

After stimulation with C-C chemokines, lymphocytes generally show a measurable intracellular Ca²⁺ flux. MIP-1 α is capable of inducing immediate transients of calcium mobilization. Typically, the levels of
20 chemokine used in these assays will be comparable to those used for the chemotaxis assays (1/1000 dilution of conditioned supernatants).

Retroviral infection assays have also been described, and recent description of certain chemokine
25 receptors in retroviral infection processes may indicate that similar roles may apply to the DC CR and/or M/DC CR. See, e.g., Balter (1996) Science 272:1740 (describing evidence for chemokine receptors as coreceptors for HIV); and Deng, et al. (1996) Nature
30 381:661-666.

V. Expression analysis of chemokine/receptor genes

RNA blot and hybridization are performed according to the standard method in Maniatis, et al. (1982)
35 Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. An appropriate fragment of a cDNA fragment is selected for use as a probe. To verify the amount of RNA loaded in each lane, the substrate membrane is reprobed with a

control cDNA, e.g., glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA (Clontech, Palo Alto CA).

Analysis of mRNA from the appropriate cell source using the probe will determine the natural size of message. It will also indicate whether different sized messages exist. The messages will be subject to analysis after isolation, e.g., by PCR or hybridization techniques.

Northern blot analysis may be performed on many different mRNA sources. However, in certain cases, cDNA libraries may be used to evaluate sources which are difficult to prepare. A "reverse Northern" uses cDNA inserts removed from vector, but multiplicity of bands may reflect either different sized messages, or may be artifact due to incomplete reverse transcription in the preparation of the cDNA library. In such instances, verification may be appropriate by standard Northern analysis.

Similarly, Southern blots may be used to evaluate species distribution of a gene. The stringency of washes of the blot will also provide information as to the extent of homology of various species counterparts.

Tissue distribution, and cell distribution, may be evaluated by immunohistochemistry using antibodies. Alternatively, in situ nucleic acid hybridization may also be used in such analysis.

A. TECK

The TECK was isolated from a RAG-1 "knockout" mouse. This animal is characterized by a great predominance of pro-T or pre-T cells, lacking more mature T cells after the block point of T cell receptor rearrangement. This suggests a role in very early T cell development, likely expressed by pro-T or pre-T cells, thymic stromal cells, and possibly macrophages, epithelial, and dendritic cells. This comports with the observation that tissue distribution studies have not detected significant expression in other organs or tissues. See also, Table 7.

Table 7: mTECK mRNA expression in tissues and cells

cDNA libraries		northern blot	
cell type or tissue	neg pos	cell type or tissue	neg
Th2 CD4+ T cells	X	heart	X
Th1 CD4+ T cells	X	brain	X
Lung	X	spleen	X
L cells	X	lung	X
RAG-1 KO lung	X	liver	X
RAG-1 KO heart	X	skeletal muscle	X
RAG-1 KO brain		kidney	X
RAG-1 KO spleen	X	testis	X
RAG-1 KO kidney	X	thymus	
RAG-1 KO testis		small intestine	
RAG-1 KO thymus		CD4+8- thymocytes R/A	X
RAG-1 KO liver		CD4-8+ thymocytes R/A	X
CD4-8- thymocytes	X	CD4-8- thymocytes R/A	X
A20-J B-cell lymphoma	X	B220+ splenocytes R/A	X
BW CD4-8-3- hybridoma	X	Thy-1+ splenocytes R/A	X
pro-T cells		1G18LA macrophages R/A	X
pre-T cells	X	primary thymic stroma R/A	X
30-R bone marrow stroma	X	3D.1 thymic epithelial R/A	X
D10 T-cell hybridoma	X	MTSC-C thymic epithelial	X
CTL T-cell clone	X	30.R bone marrow stroma	X
peritoneal macrophages	X		
splenic dendritic cells	X		

Analysis of mTECK mRNA was carried out as described. + to +++ indicates the relative intensity of the signal. R/A: resting or activated.

Species analysis indicated positive signals by hybridization in human, rat, and hamster DNA. Tissue distribution analysis suggests that the gene is expressed in human small intestine, which also is a tissue which supports T cell differentiation.

The combination of the structure and distribution of this chemokine suggests a role in T cell development, which normally occurs in the thymus.

B. MIP-3 α

The MIP-3 α was identified from a cDNA library made from human monocytes activated with LPS and IFN- γ , in the presence of anti-IL-10. See, Rossi, et al. (1997) J. Immunology 158:1033-1036, which was published after priority dates of this filing. Message of the chemokine has also been detected in pancreatic islet cells, fetal lung, and hepatic HEPG2 cells, suggesting a physiological role in inflammation or medical conditions in such organs/tissues.

The gene is expressed in HL-60 (promyelocytic leukemia); S3 (HeLa cell); K562 (chronic myelogenous leukemia); MOLT-4 (lymphoblastic leukemia); Raji (Burkitt's lymphoma); SW480 (colorectal adenocarcinoma); A549 (lung carcinoma); and G361 (melanoma) cell lines, as determined by probing on a tissue blot from CLONTECH. Tissue expression gave a positive signal in lymph node, appendix, peripheral blood lymphocytes, fetal liver, and fetal lung, suggesting a physiological role in inflammation or medical conditions in such organs/tissues; but no detectable signal in spleen, bone marrow, brain, and kidney.

The main transcript appears to be about 1.2 kb, with two additional transcript sizes in fetal lung RNA. Among the various tissues, transcript sizes of 1.8, 2.7, and 4.2 kb were detected.

Positive signals were also detected in the following cDNA libraries: dendritic cells activated with LPS, but not when activated with GM-CSF and IL-4; monocytes treated with LPS, IFN- γ , and anti-IL-10, but

not when treated with LPS, IFN- γ , and IL-10; and activated PBMC.

These expression data implicate this chemokine in inflammatory responses upon cell activation. The lymph nodes, appendix, and PBL are sites where inflammatory processes take place. The MIP-3 α may exert its effects on monocytes and cells involved in inflammatory events. Other structural features implicate this chemokine in eosinophil and lung physiology, e.g., asthma indications. Thus, an antagonist of the chemokine, e.g., an antibody, may be important for treatment of asthmatic conditions. Also, IL-10 appears to inhibit MIP-3 α expression.

The human MIP-3 α is a ligand for the DC CR. Thus, a positive control exists for the the Ca⁺⁺ flux assay for that receptor. This allows for the further screening of agonist ligands for the DC CR. Moreover, the DC CR was isolated from eosinophil cDNA, and observations have been made that eosinophils migrate to MIP-3 α in vitro. These suggest that the MIP-3 α interaction with the DC CR is important in recruitment of eosinophils, as occurs with the eotaxin ligand and the CCR3. As such, antagonists of the MIP-3 α interaction with the DC CR will likely be useful in inhibiting eosinophilia, particularly in the lung, or lung inflammation. These may accompany asthmatic or other pulmonary conditions.

Antagonists to MIP-3 α may be made either with antibodies, or other binding compositions which inhibit receptor interaction. The antibodies may be to the ligand, MIP-3 α itself, or to the binding portions of the receptor, DC CR. Muteins of the chemokine may block receptor interaction, and with a positive control, chemokine muteins may be screened for variations which compete with the wild type chemokine at various concentrations. See, e.g., Kenakin (1987)

Pharmacological Analysis of Drug-Receptor Interaction
Raven Press, NY; Arunlakshana and Schild (1959) Br. J. Pharmacol. 14:48-58; Black (1989) Science 245:486-493;

Zurawski, et al. (1986) J. Immunol. 137:3354-3360;
Zurawski and Zurawski (1988) EMBO J. 7:1061-1069;
Zurawski and Zurawski (1992) EMBO J. 11:3905-3910; Imler
and Zurawski (1992) J. Biol. Chem. 267:13185-13190.

5 C. MIP-3 β

The MIP-3 β was identified in a cDNA library made from human monocytes activated with LPS and IFN- γ , in the presence of anti-IL-10. Its distribution in other cells and tissues has not been fully determined.

10 D. Dendritic Cell Receptor for chemokine; DC CR

The DC CR was isolated from a cDNA library made from a dendritic cell cDNA library. It appears to be expressed in certain T cells, spleen cell subsets, NK cells, and other cell populations enriched in dendritic
15 cells, including CD1a⁺, CD14⁺, and CD1Aa⁺ cells. It did not give a detectable signal in TF1, Jurkat, MRC5, JY, or U937 cells.

Being found on dendritic cells, its ligand, including the MIP-3 α , may be important in attracting
20 appropriate cells for the initiation of an immune response. MIP-3 α has been shown to be a very potent chemoattractant for dendritic cells. Significant roles of the ligand and receptor in pulmonary physiology are suggested, both from the distribution of the receptor
25 and ligand. The receptor may be also present in other cells important in such responses.

E. Monocyte/Dendritic Cell Receptor for chemokine;
M/DC CR

The M/DC CR was isolated from a cDNA library made
30 from primary monocyte cells activated with LPS and IFN- γ but subtracted with known high abundance genes from those cells. The abundance of this gene is probably less than about 1% of message from those cells.

Tissue expression gave a positive signal in spleen,
35 PBL, lung, placenta, and small intestine; but no detectable signal in brain, liver, kidney, and muscle. This distribution suggests a hematopoietic role.

There appears to be one main transcript, but the existence of additional or alternatively spliced messages has not been eliminated.

Positive signals were also detected in the
5 following cDNA libraries: monocytes and dendritic cells; but signals were not detectable in CD8⁺ T cells, or in either resting or activated splenocytes, gamma-delta T cells, NK cells, or B cells. Immunohistochemistry will be performed to confirm
10 absence in the T cell and B cell compartments and to check in tonsil, particularly in view of location in spleen and placenta. The relatively restricted distribution on monocytes and dendritic cells leads both to its designation, and suggests a functional role in
15 those cell types, which are important in the initiation of immune responses through their ability to process and present antigen to T cells.

VI. Specific Characterization of TECK

20 A novel CC chemokine was identified in the thymus of mouse and human and was designated TECK as Thymus Expressed ChemoKine. TECK has weak homology with other CC chemokines and maps to mouse chromosome 8. Besides the thymus, mRNA encoding TECK was detected at
25 substantial levels in the small intestine and at low levels in the liver. The source of TECK in the thymus was determined to be thymic dendritic cells, while in contrast bone marrow-derived dendritic cells do not express TECK. The murine TECK recombinant protein
30 showed chemotactic activity for activated macrophages, dendritic cells and thymocytes. We conclude that TECK represents a novel thymic dendritic cell-specific CC chemokine which is possibly involved in T-cell development.

35 Chemokines belong to a family of small peptides (6-15 kDa) whose best described biological function is to control the migration of certain leukocyte populations to localized sites of inflammation. Baggiolini, et al. (1994) Adv. in Immun. 55:97-179; Schall and Bacon (1994)

Curr Opin Immun 6:865-873; Hedrick and Zlotnik (1996)
Curr. Opin. Immunol. 8:343-347. In the last few years
many new members of the chemokine super family have seen
the characterized. Initially, new chemokines were
5 identified through their chemotactic effects on
leukocytes (Baggiolini et al. (1994); Schall and Bacon
(1994)) and were isolated mainly from blood leukocytes
or cell lines. More recently, approaches based on the
selective cloning of secreted molecules by signal
10 sequence trap (Tashiro, et al. (1993) Science 261:600-
603; Imai, et al. (1996) J. Biol. Chem. 271:21514-21521)
or on the exploitation of public and private databases
of expressed sequence tags (EST) through bioinformatics
(Hieshima, et al. (1997) J. Biol. Chem. 272:5846-5853;
15 Patel, et al. (1997) J. Exp. Med. 185:1163-1172; and
Rossi, et al. (1997) J. Immunol. 158:1033-1036), have
allowed the rapid identification of novel chemokines
based on sequence and structural homologies. These
approaches take advantage of the fact that most of the
20 chemokines are secreted factors whose protein sequence
contain four conserved cysteines (Schall (1994) "The
Chemokines" pp. 419-460 in Thomson (eds.) The Cytokine
Handbook, Academic Press, New York. The CXC or α
chemokine family has the two first amino-terminal
25 cysteines separated by a non-conserved amino acid. In
the CC or β chemokine family, these two cysteines are
consecutive. A third type of chemokine, the C or γ
family, is represented by lymphotactin, which conserves
two cysteines (1 and 3) instead of the original four
30 (Kelner, et al. (1994) Science 266:1395-1399). Finally,
a recently identified chemokine with three amino acids
separating the first two cysteines defines a fourth CX₃C
family (Bazan, et al. (1997) Nature 385:640-644).

Interestingly, some of the new chemokines
35 discovered show a relatively restricted pattern of
expression (Imai et al. (1996); Hieshima et al. (1997)).
It is tempting to suggest that these new approaches may
lead to the discovery of tissue- or cell-specific
chemokines. In addition, new biological evidence for

important new roles of chemokines in hemopoiesis (Cook
(1996) J. Leukoc. Biol. 59:61-66; and Nagasawa, et al.
(1996) Nature 382:635-638) and the control of viral
infections including HIV (Cocchi, et al. (1995) Science
5 270:1811-1815; and Cook, et al. (1995) Science 269:1583-
1585). Thus, the molecular cloning of novel chemokines
through DNA-based strategies may uncover novel proteins
belonging to the chemokine super family but whose
physiological role goes beyond the control of
10 inflammation.

In an attempt to identify novel genes involved in
T-cell development, we analyzed a cDNA library from the
thymus of Recombinase Activation Gene-1 (RAG-1)
deficient mice. We identified a novel CC chemokine
15 designated TECK for Thymus Expressed ChemoKine, based on
sequence homology with other known chemokines. We
subsequently isolated the human homologue of TECK. The
pattern of expression of TECK mRNA is highly restricted
to the thymus and small intestine in both human and
20 mouse. Moreover, in the mouse thymus, TECK protein is
produced by dendritic cells while splenic dendritic
cells do not express TECK mRNA. Recombinant TECK
showed chemotactic activity on thymocytes, macrophages,
THP-1 cells and dendritic cells, while it was inactive
25 on peripheral lymphocytes and neutrophils. The
restricted pattern of expression of TECK together with
its biological properties suggest a role for this novel
dendritic cell-specific chemokine in T-cell development.

A. Cloning and structural analysis of mouse TECK
30 A directional cDNA library was made from RAG-1
deficient mouse thymus and analyzed by random
sequencing. One of the clones contained an open reading
frame with significant homology to previously described
CC chemokines. The full-length cDNA contains 1037 bp
35 including an open reading frame of 426 bp encoding a
protein of 142 amino acids and will be identified in
this report as mTECK (see Table 1). In the 3'
untranslated region, there is one unique polyadenylation
signal consistent with the single mRNA species observed

in northern blots. The mTECK cDNA does not contain any ATTTA transcript destabilization motif (Shaw and Kamrn (1986) Cell 46:659-667). The comparison of the amino acid sequence of mTECK with previously described murine CC chemokines demonstrates the conservation of the four cysteines present in all these chemokines. However, mTECK shows few additional identities with these proteins.

B. Cloning and molecular characterization of human TECK

To investigate the possible existence of a gene homologous to mTECK in other mammalian species, a Southern blot with genomic DNA from various species was hybridized with the mTECK cDNA probe. Under high stringency conditions, hybridizing bands were detected in mouse, rat, hamster and human genomic DNAs. Interestingly, a single band was detected in human, suggesting that a single gene encodes for TECK in this species. The multiple bands present in mouse, rat and hamster could be the result of a internal EcoRI site within the TECK gene. Alternatively, the TECK gene may have been duplicated in these species.

In order to clone the human homologue of mTECK, a blot of cDNAs from a panel of human cDNA libraries was hybridized with the mTECK cDNA probe. A signal was observed in a fetal small intestine cDNA library. Screening of this library with the mTECK probe allowed the isolation of several identical clones of 1012 bp with an open reading frame of 453 bp encoding a protein of 151 amino acids. This protein had a much higher degree of homology at the nucleic acid level (71% nucleic acid identity for the open reading frame and 49.3% amino acid identity) to mTECK than to other known CC chemokines and was thus designated as hTECK.

C. DNA sequencing and bioinformatics

The nucleotide sequence of CRAM was determined using an ABI 377 automated sequencer and standard techniques. DNA sequence analyses were performed using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI) and

MacVector 6.0 (Oxford Molecular Group). Comparisons to GenBank databases were performed using the BLAST program on web-based servers. Sequence alignments and phylogenetic analyses utilized ClustalW 1.6 (Higgins, et al. (1996) Methods in Enzymology 266:383) and TreeViewPPC 1.2 (Page (1996) Computer Applications in the Biosciences 12:357).

D. Analysis of CRAM mRNA expression

Multiple-tissue Northern blots were purchased from Clontech (Palo Alto, CA). Poly(A)+ RNA from human monocytes was used for RNA blot analysis. cDNA libraries from human cells (5 µg) in the pSPORT vector (Life Technologies) were digested with SalI and NotI to release cDNA inserts, electrophoresed on 1% agarose gels, and subjected to Southern blot transfer/hybridization. Hybridizations with ³²p-labeled CRAM DNA fragments encoding the C-terminal 144 amino acids of the predicted ORF were done at 65° C in ExpressHyb (Clontech, Palo Alto, CA) for 2 hr, followed by two stringent washes at 50° C in 0.1X SSC, 0.1% SDS for 45 min. Hybridization was detected using a STORM 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA). Reverse transcriptase PCR (RT-PCR) was performed with Superscript II reverse transcriptase (Life Technologies) and Taq DNA polymerase (Boehringer-Mannheim, Indianapolis, IN). PCR was for 35 cycles of 95° C/45 sec, 62° C/30 sec, 72° C/60 sec. Primers specific for exon 1 (5'-AGACGCTTCAGAGATCCTCTGGAGGCC; SEQ ID NO: 22) or exon 2 (5'-GAAGCTGCTTCGGGGGGTGAGCAAAC; SEQ ID NO: 23) were used in conjunction with an exon 3-specific primer (5'-CAAACACAGCAGAGCAGAGTGATGGCACC; SEQ ID NO: 24) for amplification.

E. Chromosomal localization

PCR was performed on genomic DNA from the 83 cell lines of the Stanford Human Genome Center G3 radiation hybrid panel (Research Genetics, Huntsville, AL) using CRAM primers: (5'-GTGTCCTGGCATGGGTAACAGCC; SEQ ID NO: 25) and (5'-CGGTGGAATGGTCAGGTTCTTCCC; SEQ ID NO: 26) as

previously described for the GeneBridge 4 radiation hybrid panel (Samson, et al. (1996) Genomics 36:522). Data correlating the presence or absence of PCR product to each cell line were entered into the RHserver (Stanford Human Genome Center). Co-localized STSs were identified on the human physical map using the Entrez server (National Center for Biotechnology Information).

F. Chemotactic activities of mTECK protein

To evaluate the biologic properties of mTECK, a recombinant protein with a N-terminal FLAG peptide was obtained in a bacterial expression system. In some experiments, a recombinant mTECK protein with a C-terminal FLAG was used and similar results were obtained. Interestingly, mTECK induced the migration of mouse thymocytes (Figure 1A). The optimal response was obtained with a dose of 10 ng/ml TECK. Cell migration was determined to be chemotaxis and not chemokinesis through the checkerboard analysis. Furthermore, it is established that chemokines bind to specific receptors that are coupled through heterotrimeric G proteins to intra-cellular signal-transducing pathways. Murphy (1994) Annu. Rev. Immunol. 12:593-633. To determine whether the chemotaxis of thymocytes involved a G protein-coupled receptor, cells were incubated prior to the assay with 10 ng/ml pertussis toxin which ADP-ribosylates G α_i -proteins. Katz, et al. (1992) Nature 360:686-689. This pre-treatment completely abrogated the chemotactic response of thymocytes to mTECK (Figure 1A).

The recombinant mTECK protein also induced the migration of human monocytic THP-1 cells activated for 16 hours with IFN- γ (Figure 1B), while it was not significantly active on resting THP-1 cells. This experiment showed that mTECK is active on human cells. In addition, mTECK induced activated mouse peritoneal macrophages to migrate as well as highly purified mouse splenic dendritic cells (Figure 1B). In all these experiments, the optimal dose of mTECK was 10 ng/ml. In contrast, no chemotaxis was observed with bone

marrow cells, purified neutrophils, splenic B cells, splenic T cells or IL-2 activated RAG-1 deficient mouse splenocytes lacking mature T and B lymphocytes (Mombaerts, et al. (1992) Cell 68:869-877) and
5 therefore enriched in NK cells. These data are consistent with the absence of in vivo accumulation of neutrophils, monocytes or lymphocytes 2 and 5 h following an intra-peritoneal injection of 10 μ g mTECK. Collectively, these data indicate that TECK is a
10 chemotactic factor for thymocytes, macrophages and dendritic cells.

G. TECK, a distant member of the CC chemokine family

In this report, we describe the molecular
15 isolation and characterization of TECK, a novel mouse and human CC chemokine. Analysis of its predicted amino acid sequence showed that TECK is distantly related to previously described CC chemokines. Conservation of particular amino acids among most CC
20 chemokines may be related to their functional importance. Beall, et al. (1992) J. Biol. Chem. 267:3455-3459; and Lusti-Narasimhan, et al. (1995) J. Biol. Chem. 270:2716-2721. In particular, a tyrosine residue between the second and third cysteines has been
25 shown to be critical for monocyte chemotaxis (in position 50) (Beall et al. (1992)). While TECK does not have a tyrosine at this particular position, it has one in position 52 that may have the same function, since TECK is chemotactic for activated monocytes. In
30 addition to these differences in the primary structure, the gene encoding TECK maps on chromosome 8 in the mouse, unlike most other CC chemokines which are clustered on chromosome 11. This is not the first report of an unusual chromosomal location for a CC
35 chemokine. We have cloned the human CC chemokine MIP-3 β and showed that its gene was on chromosome 9 rather than 17 (Rossi, et al. (1997)), and the gene encoding the novel human CC chemokine MIP-3 α /LARC (Rossi, et al. (1997)) has been mapped on chromosome 2 (Hieshima, et

al. (1997)). It is likely that the CC chemokines on chromosome 11 in the mouse and 17 in human have been generated through gene duplication of a primordial chemokine. Our results suggest that TECK may have been
5 generated at an earlier stage during evolution. In this regard, the TECK gene may have evolved to ensure functions similar to other CC chemokines with a distant primary structure but through similar receptor(s) as dictated by its secondary and tertiary structures.
10 Alternatively, the receptor(s) and physiological role of TECK may be unique among chemokines.

H. TECK expression and function is associated with T-cell development

We observed that TECK was strongly expressed in
15 the thymus which is the primary lymphoid organ where T-cell development takes place. Recently, another CC chemokine highly expressed in the thymus, TARC, has been identified. Imai, et al. (1996). However, TARC is also expressed in lung and colon as well as
20 activated PBMC (Imai, et al. (1996)) while TECK was absent from these tissues. Besides the thymus, numerous reports indicate that T cell development can occur in the small intestine (Poussier and Julius (1994) Annu. Rev. Immunol. 12:521-553) where TECK is
25 also expressed. Interestingly, the liver has also been suggested to support T-cell development to some extent (Abo, et al. (1994) Int. Rev. Immunol. 11:61-102) and we observed a low TECK expression in a liver cDNA library. These data show that TECK expression
30 correlates with organs that support T-cell development.

While many molecular and cellular aspects of T-cell differentiation are well documented, the precise role of chemokines in T-cell development is still unknown. Recently, it has been shown that the bone
35 marrow stroma-derived CXC chemokine SDF-1 is important for B lymphopoiesis and myelopoiesis since SDF-1 -/- mice are impaired for these functions (Nagasawa, et al. (1996)). Similarly, it is likely that chemokines act at different steps of T-cell differentiation.

Chemokines, together with the expression of appropriate adhesion molecules, may dictate the migration of uncommitted progenitors from the bone marrow to other anatomic locations. Indeed SDF-1 is chemoattractant for human CD34+ progenitor cells. Aiuti, et al. (1997) J. Exp. Med. 185:111-120. The observation that TECK is chemoattractant for thymocytes but not for mature peripheral T cells suggests that TECK could attract T-cell progenitors to the thymus. Such populations are very difficult to isolate in sufficient numbers to conduct in vitro chemotaxis experiments, but we are currently designing new strategies to address this important question. In addition, we have not found significant chemotactic activity of TECK on bone marrow cells. SDF-1 was shown to be much less potent on CD34+ progenitors from the peripheral blood than those from the bone marrow. Aiuti, et al. (1997). It is possible that the sensitivity of progenitor cells to TECK would increase as these cells leave the bone marrow to colonize lymphoid organs. Importantly, intra-thymic maturation is also characterized by a directional migration from the subcapsular region which contains the earliest progenitors to the cortex and finally to the medulla where thymocytes finish their maturation (Boyd, et al. (1993)). It is possible that the secretion of TECK by medullary dendritic cells may play a role in this directional migration. Yet another possibility is that TECK may play a role in the organization and development of the thymic stroma.

We also showed that TECK is chemotactic for activated macrophages and dendritic cells. These two cell types also play important roles in T-cell development. Through a complex screening process involving positive and negative selection events most of the antigenic specificities randomly generated in the thymus will be eliminated by programmed cell death (Janeway (1994) Immunity 1:3-6). The efficient scavenging of dead thymocytes is probably mediated, at least partially, by thymic macrophages and thus TECK

could play an important role through its action on activated macrophages. Further along, T-cells with a high affinity for self-antigens and thus potentially harmful are eliminated through negative selection (Janeway (1994)). It is believed that thymic dendritic cells are primarily responsible for the negative selection of thymocytes, therefore playing a major role in the establishment of tolerance. Inaba, et al. (1991) J. Exp. Med. 173:549-559. An efficient mechanism of central tolerance should eliminate T cells potentially reactive against auto-antigens which are not expressed in the thymus, such as organ specific auto-antigens. Several known chemokines induce the migration of dendritic cells and could therefore contribute to their recruitment during peripheral immune responses. Sozzani, et al. (1995) J. Immunol. 155:3292-3295; and Xu, et al. (1996) J. Leukoc. Biol. 60:365-371. Similarly, dendritic cells presenting organ-specific or other antigens could be recruited to the thymus or the small intestine and induce negative selection of T cells specific for these antigens. It is possible that thymus- and small intestine-specific chemokines active on dendritic cells such as TECK could play an important role in the establishment of tolerance. Thus, TECK could potentially interact at several important steps of T-cell development. Future experiments will aim to define the precise role of TECK in T-cell development and other physiological processes through the use of genetically modified mice.

I. TECK is specifically expressed by thymic dendritic cells

Dendritic cells represent an heterogeneous cell population derived from bone marrow progenitors. They are present in non-lymphoid organs as immature dendritic cells (such as Langerhans cells in the skin) where they display a high ability for antigen capture. Cella, et al. (1997) Curr. Opin. Immunol. 9:10-16. Subsequent to antigen challenge, they will migrate to secondary lymphoid organs and will acquire a high

capacity to present processed antigens to naive T-cells to initiate a specific immune response (Cella, et al. (1997)). It has been shown that dendritic cells can derive from CD34+ progenitors cultured in the presence of GM-CSF and TNF- α (Caux, et al. (1992) Nature 360:258-261; and Caux, et al. (1996) J. Exp. Med. 184:695-706) or from monocytes in the presence of GM-CSF and IL-4 (Sallusto and Lanzavecchia (1994) J. Exp. Med. 179:1109-1118). Interestingly, there is also evidence for a lymphoid dendritic cell precursor in thymus and bone marrow which is able to derive both lymphocytes and dendritic cells in the absence of GM-CSF. Ardavin, et al. (1993) Nature 362:761-763; Galy, et al. (1995) Immunity 3:459-473; Marquez, et al. (1995) J. Exp. Med. 181:475-483; and Wu, et al. (1996). These lymphoid-derived dendritic cells may have different functional properties such as a negative regulation of T-cell responses since they express FasL in the mouse. Suss and Shortman (1996) J. Exp. Med. 183:1789-1796. We found that TECK was expressed at high levels in mouse thymic dendritic cells but was absent in cDNA libraries from mouse splenic dendritic cells or from human dendritic cells generated in vitro from CD34+ precursors or monocytes. Interestingly, mTECK mRNA was present at a low level in a population of early thymocyte progenitors still able to derive dendritic cells (Wu, et al. (1996). Thus, it would be tempting to suggest that TECK could be a specific marker of lymphoid-derived dendritic cells. However, we observed that TECK was absent from splenic dendritic cells that likely contain lymphoid-derived dendritic cells. The expression of TECK mRNA appeared in the spleen of mice injected with LPS would suggest that peripheral dendritic cells may express TECK upon activation, but we found that TECK was not expressed in cDNA libraries of bone-marrow derived dendritic cells activated with LPS, PMA and ionomycin or IL-1 α and TNF- α . It is possible that the normal expression of TECK is specific for lymphoid-derived dendritic cells or,

alternatively, that it is upregulated by very specific stimuli present in the thymic and intestinal micro-environment under physiological conditions. Consistent with the latter hypothesis is our observation of
5 specific staining of thymic endothelial cells with anti-TECK antibody since we have not been able to find TECK expression in human HUVEC endothelial cells by northern blot analysis, without activation or following a 16 hour-activation with various combinations of IL-1,
10 TNF- α , IL-4, IL-7 and oncostatin while some of these stimuli induce the expression of other CC chemokines in endothelial cells. Rollins and Pober (1991) Am. J. Pathol. 138:1315-1319; Marfaing-Koka, et al. (1995) J. Immunol. 154:1870-1878; Garcia-Zepeda, et al. (1996) J. Immunol. 157:5613-5626; and Garcia-Zepeda, et al.
15 (1996) Nat. Med. 4:449-456. Taken together, our data strongly suggest that TECK is a novel chemokine specifically expressed by activated lymphoid-derived dendritic cells.

20 Through their function of antigen presentation, dendritic cells play major roles in the establishment of tolerance and in the initiation of an antigen-specific immune response. The use of purified dendritic cells has been recently proposed in different
25 therapeutic protocols (Cella, et al. (1997)). The discovery of factors with a regulated expression in dendritic cells such as the novel CC chemokine TECK will certainly improve our knowledge of the biology of dendritic cells and lead to the design of relevant in
30 vivo applications.

J. Mice and in vivo experimental procedures

Four to eight week-old and time-pregnant BALB/c mice were purchased from Simonsen Laboratories (Gilroy, CA). RAG-1-deficient mice (Mombaerts, et al. (1992))
35 were purchased from The Jackson Laboratory (Bar Harbor, ME). To analyze TECK expression after in vivo activation, various organs were recovered from pools of 2 mice 3 hours after intravenous LPS injection (50 μ g LPS in 200 μ l PBS or 200 μ l PBS for controls).

K. Cell purification, culture and stimulation.

THP-1 cells (TIB-202 from the American Type Culture Collection, Rockville, MD) were cultured in complete medium which consisted in RPMI 1640 medium
5 (JRH BioSciences, Lenexa, KS) supplemented with 10% FCS, 200 mM L-glutamin, 5×10^{-5} M mercaptoethanol, MEM amino-acids and vitamins, sodium bicarbonate, penicillin, streptomycin (all from Sigma, ST. Louis, MO), and gentamycin (Boehringer, Indianapolis, IN). To
10 obtain activated mouse macrophages, 10 ml of cold PBS were injected into the peritoneum and the collected cells allowed to adhere to plastic for 24 h in complete medium. The adherent fraction, mostly macrophages, was then collected. To obtain splenic dendritic cells, a
15 splenocyte cell suspension was prepared in RPMI 1640 Dutch modified medium (Life Technologies, Paisley, Scotland) as described previously in, e.g., Macatonia, et al. (1987) J. Exp. Med. 166:1654-1667. Splenocytes were incubated at 37° C for 16 h and the cell
20 suspension was collected and laid over Metrizamide (Nycomed Pharma, Oslo, Norway). After centrifugation for 10 min. at 1700 x g, the low interface was collected and stained with anti-Mac-1 (Pharmingen, San Diego, CA) and the anti-CD11c N-418 antibodies
25 (Macatonia, et al. (1993) J. Immunol. 150:3755-3765). Splenic dendritic cells were sorted by flow cytometry on a FACStar plus cell sorter (Becton Dickinson, Mountain View, CA) to a purity greater than 98% upon reanalysis in all the experiments included in this
30 report. To obtain thymic dendritic cells, thymuses were cut in small fragments and resuspended in 10 ml of RPMI-1640 +10% FCS containing 1 mg/ml collagenase and 0.02 mg/ml DNase I (both from SIGMA) and digested with continuous agitation at room temperature for 30 min.
35 (Shortman, et al. (1995) Adv. Exp. Med. Biol. 378:21-29). One ml of 0.1M EDTA pH 7.2 was added for an additional 5 min. Cells were then washed in complete medium, resuspended in complete medium and overlaid onto Metrizamide. The thymic dendritic cell-enriched

preparation was then stained with anti-IAd and N-418 antibodies and the dendritic cells sorted by flow cytometry

L. Molecular cloning of mouse and human TECK

5 The cDNA encoding mouse TECK was obtained by random sequencing of a RAG-1 KO mouse thymic directional cDNA library. Briefly, mRNA was extracted using RNazolTM B (Tel-Test, Friendswood, TX) and then oligotex-dT mRNA kit (Quiagen, Chatsworth, CA) following the manufacturer's instruction. A directional cDNA library was prepared using the SuperscriptTM Plasmid System (Gibco-BRL, Grand Island, NY) and cloned into the pME18s plasmid vector. Sequencing was done using the TaQ DyeDeoxy Terminator
10 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). To determine whether TECK was present in other mammals including human, a Southern blot containing EcoRI digested genomic DNA from different species (Bios Laboratories, New Haven, CT) was hybridized with the
15 full-length mouse TECK cDNA.
20

 The cDNA encoding human TECK was found by screening of a small intestine cDNA library using the full-length mouse TECK cDNA as a probe following standard procedures.

25 M. Northern blot analysis of RNA and Southern blot analysis of cDNA libraries

 All RNA's were isolated from tissues or cells using RNazolTM B (Tel-Test) and analyzed after electrophoresis in a 1% formaldehyde-agarose gel (10
30 µg/lane). RNA's were then blotted onto a Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL). Some northern blots of mRNA were bought from Clontech (Palo Alto, CA). To analyze the expression of TECK in cDNA libraries (obtained from T. MacClanahan, DNAX), 10 µg
35 of DNA were digested with the appropriate restriction enzymes to release their inserts and analyze by Southern blotting onto nylon membranes. Northern blots and blots of cDNA libraries were hybridized for 16 hours at 65°C with a ³²P-labeled probe consisting in

the full-length cDNA encoding for mouse or human TECK and then washed and exposed, according to standard protocols.

N. Inter specific mouse backcross mapping

5 Inter specific backcross progeny were generated by mating (C57Bl/6J x M. spretus) F1 females and C57Bl/6J males as described, e.g., in Copeland and Jenkins (1991) Trends Genet. 7:113-118. A total of 205 N₂ mice were used to map the Teck locus. DNA isolation, 10 restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization with the full-length mTECK cDNA probe were performed as described, e.g., in Jenkins, et al. (1982). Fragments of 7.5, 6.9, and 2.5 kb were 15 detected in HincII digested C57Bl/6J DNA and fragments of 8.8 and 5.4 kb were detected in HincII digested M. spretus DNA. The presence or absence of the 8.8 and 5.4 kb HincII M. spretus-specific fragments, which cosegregated, was followed in backcross mice. A 20 description of the probes and RFLPs for two of the loci linked to Teck including Insr has been reported previously, e.g., in Ceci, et al. (1990) Genomics 6:72-79. Recombination distances were calculated as described (Green (1981) "Linkage, recombination and 25 mapping" pp. 77-113 in Genetics and Probability in Animal Breeding Experiments, Oxford University Press, New York) using the computer program SPRETUS MADNESS.

O. Measurement of TECK mRNA expression by RT-PCR
30 RNA's from sorted thymic dendritic cells or fetal thymuses were prepared with the RNeasy total RNA kit (Quiagen, Chatsworth, CA), following the manufacturer's instructions. First strand cDNAs were generated by reverse transcription with a random hexamer in a 10 µl reaction and 1 µl of this reaction was used as a 35 template for PCR. TECK expression was compared to the expression of hypoxanthine-guanine phosphoribosyl transferase (HPRT). Primer sequences were as follows: TECK: 5' primer, 5'CCTTCAGGTATCTGGAGAGGAGATC3' (SEQ ID NO: 20; nucleotides 58-72 of SEQ ID NO: 1) and 3'

primer, 5'CACGCTTGTTACTGTTGGGGTTC3' (SEQ ID NO: 21; complement of nucleotides 447-468 of SEQ ID NO: 1), HPRT: 5' primer, 5'GTAATGATCAGTCAACGGGGGAC3' (SEQ ID NO: 17) and 3' primer, 5'CCAGCAAGCTTGCAACCTTAACCA3' (SEQ ID NO: 18). Samples were submitted to 25 cycles of amplification, each composed of 94° C for 1 min., 57° C for 30 s and 72° C for 2 min. PCR products were then separated by electrophoresis in 2% agarose gels and stained with ethidium bromide.

10 P. In Situ Hybridization

Biotin-14-CTP labeled sense and antisense riboprobes were generated using a non radioactive RNA labeling system (Gibco, Gaithersburg, MD) and the plasmid PCRII (InVitrogen, Carlsbad, CA) containing a 15 400 base pair TECK cDNA fragment inserted by PCR and TA cloning (InVitrogen). Paraffin-embedded tissues were cut in 3-5 µm sections, mounted on slides, baked at 60° C for one hour, deparaffinized in xylene (Fisher Scientific, Pittsburgh, PA) and immersed in 100% 20 ethanol. Sections were then incubated for 10 min at 37° C in proteinase K solution (40 mg/ml) (Gibco) in PBS and rinsed for 2 min in PBS at room temperature before being refixed in 10% formalin (Fisher Scientific, Pittsburgh, PA) in PBS for 1 min. Next, 25 the sections were dehydrated through graded solutions of ethanol and air dried. Hybridization was carried out using the Gibco in situ hybridization and detection system kit. Vanadyl ribonucleoside complex (Gibco) was added to the hybridization solution (39 mM final). A 30 0.1 µg/ml concentration of each probe was used during an 18 h hybridization at 42° C. Post-hybridization washes used room temperature 0.2X SSC. Following detection and substrate visualization, the slides were counterstained with 1% nuclear red stain (Sigma, St. 35 Louis, MO).

Q. Immunohistochemistry

A polyclonal antibody specific of a synthetic decapeptide identical to the C-terminus part of murine TECK (Figure 1) was prepared in rabbits by Research

Genetics (Huntsville, AL). Normal rabbit serum from a pool of 50 different animals (Research Genetics) was used as a negative control. To study TECK protein expression in the mouse thymus, 6 μ m thick cryostat sections were thaw mounted on organosilicone subbed slides (American Histology Reagent Co., Stockton, CA.) and fixed in 3% formalin (Fisher Scientific, Springfield, NJ) in Hank's Balanced Salt Solution with 0.01M HEPES (HBSS-HEPES), pH 7.4, for 15 min at room temperature. The sections were sequentially blocked for endogenous biotin binding using the Vector blocking kit (Vector Laboratories, Burlingame, CA) and for endogenous peroxidase activity with a 1% hydrogen peroxide, 0.2M sodium azide solution, in HBSS-HEPES with 0.1% saponin (staining buffer). Non-specific antibody binding sites were then blocked with 10% normal goat serum (Sigma) in staining buffer. Sections prepared as above were first incubated for 18 h at 25°C with 1/500 dilution of polyclonal antibody or control rabbit serum in staining buffer. In the second step, the sections were incubated for 1 h at room temperature with biotin labeled goat anti-rabbit IgG (2 μ g/ml) (Vector Laboratories) in staining buffer and then for 30 min at room temperature with the Vectastain Elite ABC Kit (Vector Laboratories) in staining buffer. The sections were then rinsed in HBSS-HEPES without saponin. Immunoenzyme tissue staining was revealed with 3, 3' - diaminobenzidine tetrahydrochloride (DAB) substrate (0.5 mg/ml) (Sigma) in 0.05M Tris, pH 7.4, containing 0.0075% hydrogen peroxide. The substrate reaction was stopped by rinsing the sections in distilled water. The sections were then counterstained with Harris' hematoxylin (Shandon Lipshaw, Pittsburgh, PA).

The expression of TECK mRNA in murine adult thymus was analyzed by in situ hybridization and revealed a discrete positive non-lymphoid population within the thymus medulla. The expression of TECK protein was analyzed by using a polyclonal anti-serum made in a rabbit immunised with a peptide that consisted in the

last 12 amino-acid of the murine TECK protein sequence. This polyclonal antibody reacts with the murine TECK recombinant protein prepared at DNAX both in ELISA and western blot. The application of this anti-serum on
5 mouse adult thymic sections confirmed the distribution pattern obtained by in situ hybridization: the cells producing TECK are medullary stromal cells. The precise cell type producing TECK within the mouse thymus was identified, using the same anti-serum on sorted thymic
10 subsets, as being the thymic dendritic cells.

R. Production of recombinant mouse TECK in
Escherichia coli and other chemokines

Mouse recombinant TECK was produced in E. coli as a N-terminal FLAG (DYKDDDDKL; SEQ ID NO: 19) fusion
15 protein. Briefly, the fusion construct containing FLAG followed by the mTECK sequence minus the leader peptide (see Table 1) was obtained by PCR amplification of the TECK cDNA in order to flank the coding sequence with HindIII and EcoRI sites and subsequent ligation in the
20 pFLAG.1 vector which contains the FLAG sequence and an OmpA signal sequence. Electro-competent UT 4400 E. coli were transformed with the pFLAG.1-mTECK plasmid. The cells were grown in 2 x LB plus 50 µg/ml Ampicillin, induced at an OD. of 2.3 with 400 µM IPTG and harvested.
25 The cell pellet was resuspended in cold lysis buffer (20 mM Tris pH 8, 2 mM EDTA, 20% sucrose, 0.1 mg/ml lysozyme, 100 µl Benzonase), homogenized and allowed to sit for 30 min. Then the same amount of a 1:4 dilution of cold lysis buffer without lysozyme was added for 10
30 more min. The solution was spun and the supernatant was filtered through a 0.2 µm membrane and then diluted 1:1 in 50 mM Tris pH 7.5. The diluted osmotic extract was submitted to chromatography on a Q-sepharose column equilibrated with 50 mM Tris pH 7.5 and eluted with a
35 linear salt gradient. The fractions containing the recombinant protein were pooled. The fractions were then loaded onto a S-sepharose column equilibrated with 20 mM acetate pH 4.0. The column was eluted with a linear salt gradient and then with a 1.5M NaCl wash that

contained the protein. Finally, the eluate was loaded onto a reverse phase column. The column was eluted with a linear gradient of 20% to 80% acetonitrile + 0.1% TFA. The concentration of the mTECK protein was estimated by
5 Comassie blue staining and densitometric scanning of a 10% Nu-PAGE gel with lysozyme as a standard. The purity was estimated at 100% by sequencing of the N-terminus of the recombinant protein. Recombinant murine MIP-1 α (R&D Systems, Minneapolis, MN) and lymphotactin
10 (Hedrick, et al. (1997) J. Immunol. 158:1533-1540) were used as controls.

S. Assay for chemotaxis

The in vitro migration of cells isolated as described above in response to TECK or other factors was
15 assessed in a modified Boyden micro chamber (Neuroprobe, Cabin John, MD) as described previously (Kelner, et al. (1994)). Briefly, factor dilutions in DMEM medium (Gibco) were loaded in the lower compartment in duplicate and 10⁵ cells in a 50 μ l volume of DMEM were
20 loaded in the upper compartment. The two compartments were separated by a 5- μ m or 8- μ m pore size polycarbonate filter (Nucleopore, Pleasanton, CA). After incubation at 37° C for 80 min (or 120 min for lymphocytes), the filters were fixed in methanol and stained with Fields A
25 and B. Cell migrated on the other side of the membrane were counted per five high-power fields (100 x) under microscope. The chemotactic index was calculated from the number of cells counted with the test sample divided by the number of cells counted with medium alone.

30 Northern blot analysis was performed of RNA from different organs hybridized with the mTECK cDNA probe with or without in vivo LPS stimulation. Hybridizing bands corresponded to the predicted \approx 1040 bp size for mTECK mRNA. Significant induction occurred in spleen
35 (with virtually no background), and in thymus and small intestine (both with higher background); no signal was detected in either condition for heart, lung, kidney, or liver.

mTECK mRNA expression was analysed in the mouse fetal thymus. RNA's from fetal thymic lobes were extracted at day 14, 15, 16 and 17 of gestation. Positive RT-PCR signals were detected in each of day 14, 125, 16, and 17 samples.

mTECK mRNA expression in thymic dendritic cells was evaluated. A population enriched in thymic dendritic cells was prepared from 15 pooled adult thymuses. >99% pure dendritic cells were then sorted by flow cytometry based on their MHC Class II+ N-418+ phenotype. mTECK mRNA was then analyzed by RT-PCR and a MHC class II+ N-418- population sorted in the same experiment was used as a negative control. The N418+ sample gave a positive signal, while the N418- sample did not.

Expression analysis was performed with hTECK mRNA in different Human Tissues and Cell Types. Southern blots of human cDNA libraries digested with the appropriate restriction enzymes were hybridized with the hTECK cDNA probe. A major band hybridizing corresponding to the predicted length of hTECK mRNA (≈ 1040 bp) was observed with sometimes some other bands that may represent incomplete cDNAs. Positive signals were detected in tonsil, fetal spleen, and fetal small intestine. No signal was detected in activated (with PMA and ionomycin for 12 h) NK cells, activated (anti-CD40 antibody and IL-4 for 6 and 12 h) splenocytes, $\gamma\delta$ T cells, activated (with anti-CD3 and PMA for 6, 12, and 24 h) PBMC, fetal testis, C+ (elutriated monocytes cultured with IFN- γ and IL-10) monocytes, C- monocytes, 70% pure dendritic cells (CD1 α + dendritic cell population obtained by expansion of CD34+ bone marrow cells with GM-CSF and TNF- α and resting), and DC3 (similar dendritic cell population stimulated with PMA and ionomycin for 1 and 6 h), DC5 (dendritic cells obtained by culturing peripheral blood monocytes in the presence of IL-4 and GM-CSF), U937 (premonocytic cell line), and CD1 α cell lines. Ras KO mouse cDNA again confirmed that the mouse and human genes crosshybridize.

Four independent lines of transgenic mice expressing TECK in the brain have been made. All animals had neurologic disorders. In addition, several of them suffered severe infections. The consequences of TECK could be a direct one on brain cells which nature remains to be identify. Alternatively, since TECK has been shown in vitro to have effects on macrophages and dendritic cells which are critical effectors of immune responses, the overproduction of TECK could lead to distant effects on these cells at sites of infection. These results suggest that the blockade of TECK production in vivo may help to resolve particular pathological processes, in particular infections. The localization suggests a physiological role in immunological responses involving the thymus, or in colon/small intestine or gastrointestinal inflammation, e.g., Crohn's disease or inflammatory bowel disease.

VII. Specific Characterization of the M/DC CR (CRAM)

Abbreviations: BAC, bacterial artificial chromosome; bp, base pair; CKR, chemokine receptor; EST, expressed sequence tag; GPR, G-protein-linked receptor; PBMC, peripheral blood mononuclear cells; STS, sequence tagged site.

We describe a novel human gene with high homology to CC- or β -chemokine receptors (CKRs). This putative CKR, CRAM, is most similar to human CCR1, with 46% amino acid identity and 65% similarity. CRAM is encoded by at least two alternatively spliced 1.5 and 1.8 kb mRNAs which specify at least two proteins differing by 12 amino acids at the N-terminus (CRAM-A and CRAM-B). CRAM mRNA was detected mainly in lymphoid tissues and expressed in activated monocytes, but not in B- or T-lymphocytes. CRAM mRNA expression was increased upon stimulation with IFN γ and LPS but was not detectably inhibited by interleukin-10. CRAM was localized to the β -CKR cluster at chromosome 3p21 and physically linked to the CCR2 and CCR5 genes. In view of its similarity and genomic linkage to β -CKRs and restricted expression

pattern, CRAM may play an important role in immune function. The existence of CRAM with alternative N-termini suggests a mechanism for altering ligand specificity and possibly signalling capacity of a single
5 CKR.

Chemokines play critical roles in the chemoattraction and activation of leukocytes (Premack and Schall (1996) Nat Med 2:1174; Murphy (1996) Cytokine Growth Factor Rev 7:47; and Furie and Randolph (1995) Am
10 J Pathol 146:1287), and have been divided into four families, based on the spacing of the first two of (usually) four conserved cysteine residues. The α chemokines, with a C-X-C motif, include IL-8, MIP-2 α , GRO β , and ENA-78. The β chemokines (C-C motif), include
15 MIP-1 α , MCP-1, TARC, and RANTES. Recently, two new chemokine families have been defined by lymphotactin (γ) and CX₃Ckine (δ). Lymphotactin has only a single cysteine residue at the corresponding location for the C-C or C-X-C motif. Kelner and Zlotnik. (1995) J Leukoc
20 Biol 57:778; Kennedy, et al. (1995) J Immunol 155:203. CX₃Ckine contains two cysteines separated by three intervening amino acids, and is tethered to the cell membrane via a long carboxy-terminal tail of mucin-like repeats. Bazan, et al. (1997) Nature 385:640.

25 Receptors for chemokines (CKRs) are G-protein coupled receptors (GPRs) with seven transmembrane domains. Novel CKRs have been identified by expression cloning of receptors binding a particular chemokine ligand (Holmes, et al. (1991) Science 253:1280) or
30 mediating HIV fusion (Feng, et al. (1996) Science 272:872), by PCR using degenerate primers specific for conserved regions (Meyer, et al. (1996) J Biol Chem 271:14445; Ponath, et al. (1996) J Exp Med 183:2437; Daugherty, et al. (1996) J Exp Med 183:2349; Kurihara
35 and Bravo (1996) J Biol Chem 271:11603; Power, et al. (1995) J Biol Chem 270:19495; Napolitano, et al. (1996) J Immunol 157:2759; and Raport, et al. (1996) J Leukoc Biol 59:18), and by random sequencing efforts followed by sequence analysis. While nearly 30 CKR-like genes

have been cloned from mammals and mammalian viruses, only 17 have been shown to bind identified chemokines. Thus, a substantial number of CKR-like molecules remain "orphan receptors." Most CKRs with experimentally
5 identified ligands bind to more than one ligand. IL-8 receptor B (CXCR2) binds to the α chemokines IL-8, NAP-2, and MGSA (Suzuki, et al. (1994) J Biol Chem 269:18263), whereas human CCR5 binds the β chemokines RANTES, MIP-1 α , and MIP-1 β (Raport, et al. (1996) J Biol
10 Chem 271:17161; and Alkhatib, et al. (1996) Science 272:1955).

We have used cDNA library subtraction to isolate genes which are induced by monocyte activation. We thereby isolated a cDNA clone from a subtracted library
15 enriched for monocyte activation-specific cDNAs that shows considerable homology to CC- or β -CKRs and maps within the β -CKR cluster on human chromosome 3p21. Expression of this gene was detected in several lymphoid tissues and in activated monocytes (but not
20 lymphocytes). We provisionally designate this gene CRAM, for chemokine receptor of activated monocytes. CRAM is expressed as at least two alternatively spliced mRNAs encoding CKRs with different N-terminal amino acid sequences, suggesting a possible novel mechanism for
25 regulation of CKR ligand specificity.

A. Cell cultures and cDNA library construction

Human PBMC were purified by density gradient centrifugation on Ficoll (Pharmacia Biotech Inc., Piscataway, NJ) using standard procedures. Monocytes
30 were enriched from PBMC by adherence to tissue culture flasks and cultured in DMEM + 10% FCS. Monocytes were activated by culture with 100 ng/ml IFN γ (R & D Systems Inc., Minneapolis, MN) and 1 μ g/ml LPS (Life Technologies, Grand Island, NY) for 1-15 hr. Total RNA
35 was prepared by guanidinium isothiocyanate lysis followed by poly(A)+ RNA selection using the OLIGOTEX kit (QIAGEN Inc., Chatsworth, CA). cDNA libraries containing $>2 \times 10^6$ independent clones were constructed using the SuperScript cDNA Kit (Life Technologies).

B. cDNA library subtractions

Subtracted cDNA libraries (activated monocytes minus resting PBMC) were constructed. See, e.g., Hara, et al. (1994) Blood 84:189; and Kennedy, et al. (1996) J Interferon Cytokine Res 16:611. The major cDNA species present in the subtracted library were then added (1 μ g each) to the resting PBMC cDNA library (150 μ g); this mixture was used as the driver cDNA for a second round of subtraction using 5 μ g of the activated monocyte cDNA library to enrich for induction-specific cDNAs which were less abundantly expressed.

C. DNA sequencing and bioinformatics

The nucleotide sequence of CRAM was determined using an ABI 377 automated sequencer and standard techniques. DNA sequence analyses were performed using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI) and MacVector 6.0 (Oxford Molecular Group). Comparisons to GenBank databases were performed using the BLAST program on web-based servers. Sequence alignments and phylogenetic analyses utilized ClustalW 1.6 (Higgins, et al. (1996) Methods in Enzymology 266:383) and TreeViewPPC 1.2 (Page (1996) Computer Applications in the Biosciences 12:357).

D. Analysis of CRAM mRNA expression

Multiple-tissue Northern blots were purchased from Clontech (Palo Alto, CA). Poly(A)+ RNA from human monocytes was used for RNA blot analysis. cDNA libraries from human cells (5 μ g) in the pSPORT vector (Life Technologies) were digested with SalI and NotI to release cDNA inserts, electrophoresed on 1% agarose gels, and subjected to Southern blot transfer/hybridization. Hybridizations with 32 P-labeled CRAM DNA fragments encoding the C-terminal 144 amino acids of the predicted ORF were done at 65° C in ExpressHyb (Clontech, Palo Alto, CA) for 2 hr, followed by two stringent washes at 50° C in 0.1X SSC, 0.1% SDS for 45 min. Hybridization was detected using a STORM 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA). Reverse transcriptase PCR (RT-PCR) was performed with

Superscript II reverse transcriptase (Life Technologies) and Taq DNA polymerase (Boehringer-Mannheim, Indianapolis, IN). PCR was for 35 cycles of 95° C/45 sec, 62° C/30 sec, 72° C/60 sec. Primers specific for
5 exon 1 (5'-AGACGCTTCAGAGATCCTCTGGAGGCC; SEQ ID NO: 22) or exon 2 (5'-GAAGCTGCTTCGGGGGGTGAGCAAAC; SEQ ID NO: 23) were used in conjunction with an exon 3-specific primer (5'-CAAACACAGCAGAGCAGAGTGATGGCACC; SEQ ID NO: 24) for amplification.

10 E. Chromosomal localization

PCR was performed on genomic DNA from the 83 cell lines of the Stanford Human Genome Center G3 radiation hybrid panel (Research Genetics, Huntsville, AL) using CRAM primers: (5'-GTGTCCTGGCATGGGTAACAGCC; SEQ ID NO:
15 25) and (5'-CGGTGGAATGGTCAGGTTCTTCCC; SEQ ID NO: 26) as previously described for the GeneBridge 4 radiation hybrid panel (Samson, et al. (1996) Genomics 36:522). Data correlating the presence or absence of PCR product to each cell line were entered into the RHserver
20 (Stanford Human Genome Center). Co-localized STSs were identified on the human physical map using the Entrez server (National Center for Biotechnology Information).

F. cDNA cloning of CRAM

We employed subtractive hybridization to identify
25 genes induced in monocytes upon activation by IFN γ and LPS. An activated monocyte cDNA library was first subtracted against a resting PBMC cDNA library. Seven prominent induced cDNAs thus identified were mixed with the resting PBMC library, which was then used as
30 "driver" in another subtraction to generate a new library containing less abundantly expressed, induction-specific cDNAs. More than 100 clones were isolated from this second-round subtracted library, representing 55 unique cDNAs, 25 of which did not correspond to known
35 cDNAs from the non-redundant section of GenBank. One of these clones contained a 1.5 kb insert encoding a large open reading frame with strong homology to all five known human β -CKRs. We designated this cDNA CRAM (chemokine receptor of activated monocytes; or M/DC CR).

G. Sequence analysis of CKRs

A phylogenetic analysis of CKRs and related gene sequences revealed two major clades or phylogenetic groups, with several receptors remaining unclustered outside these two groups. Interestingly, the two groups correlated with known ligand specificities: the α -CKR IL-8RA, IL-8RB, and fusin cluster in a single clade, while β -CKR CCR1 through CCR5 all cluster in a second clade. Of the seven receptors that do not fall into either group, one (DARC) is a promiscuous CKR that binds several α - and β -chemokines (Neote, et al. (1993) J Biol Chem 268:12247).

The 1536 bp CRAM cDNA encodes an ORF with a predicted size of about 356 amino acids. Phylogenetic analysis showed that CRAM was most closely related to β -CKRs, exhibiting strongest homology to CCR1 (46% identity and 65% similarity), and the least to CCR4, with only 33% identity and 48% similarity. Three other human orphan receptors V28, TER1, and GPR5 also group with β -CKRs, and like CRAM, may be receptors for known or yet to be identified β chemokines.

The two most highly conserved regions among CCR1 through CCR5 are in transmembrane region 2 (YLLNLAISDLLF; "TM2") and immediately after transmembrane region 3 (IDRYLAIVHAVF; "DRY-box"). These two 12-amino acid segments are invariant among CCR1 through CCR4; CCR5 shares 22 of these 24 residues. These regions are sometimes conserved among other mammalian GPR and have been used for degenerate primer PCR to clone new CKRs. CRAM is divergent in these regions (9 out of 12 amino acids in TM2; 4 out of 12 in the DRY-box), which may explain why such approaches have failed to identify CRAM. The DRY-box is in one of the three intracellular loops, and is thought to play a role in binding to heterotrimeric G proteins (Damaj, et al. (1996) FASEB J 10:1426). Because of the divergence of CRAM from the other β -CKRs in these regions, it may interact with a different subset of G protein subunits,

possibly transducing a signal different from that induced via other β -CKRs.

While human CKR genes have been localized to several different chromosomes, the β -CKR genes CCR1, CCR2, CCR3, and CCR5 all cluster in a 350 kb region at chromosome 3p21.3 (Samson, et al. (1996) Genomics 36:522). CCR4 and the orphan receptors *TER1* and *GPR5* are also located in this 3p21 region Napolitano, et al. (1996) J Immunol 157:2759; Samson, et al. (1996) Genomics 36:522; Heiber, et al. (1995) DNA Cell Biol 14:25). We determined the chromosomal location of CRAM. The Stanford G3 panel of radiation hybrids was used as templates for PCR reactions with CRAM-specific primers. Among the 83 different hybrids, 11 contained the CRAM gene as assessed by PCR. CRAM co-localized with STS D3S3888, which is located at chromosome 3p21.3. Confirmation of this result was obtained from the recently completed sequence of the 143 kb BAC clone 110p12 from the 3p21 region (GenBank accession U95626); this BAC contains the loci CCR2, CCR5, and CRAM.

A related but different CRAM cDNA was also isolated from an activated monocyte library by random sequencing. Comparing the two forms of CRAM to the genomic sequence revealed the existence of two short exons (corresponding to 95750-96064 bp and 96186-96256 bp on BAC 110p12), followed by a large third exon (96630-98093 bp) that contains almost the entire ORF for CRAM. These two CRAM cDNAs consist of either exon 2 and exon 3 (1536 bp), or exon 1 and exon 3 (1780 bp). Exon 2 contributes 12 amino acids in frame with exon 3 to form the entire 356 residue polypeptide (CRAM-A). As exon 1 has no methionine in frame with the ORF in exon 3, the translated protein from this splice variant would start with Met-13, resulting in an N-terminally truncated protein of 344 amino acids (CRAM-B).

H. Expression of CRAM mRNA

RNA blot analysis showed expression largely restricted to lymphoid tissues. Prominent expression of CRAM mRNA was observed in spleen, lymph node, thymus,

bone marrow, and fetal liver. Very little expression was detected in brain, liver, muscle, kidney, pancreas, or PBL, with moderate signals in heart, placenta, lung, and appendix. This pattern of expression was similar to
5 that of the CKR-like gene *TER1* (Napolitano, et al. (1996) J Immunol 157:2759), but quite different from the related orphan receptor genes *V28* and *blr1* (Forster, et al. (1996) Cell 87:1037; and Raport, et al. (1995) Gene 163:295).

10 Data from various hematopoietic cell types showed no evidence for CRAM expression in resting or activated lymphocytes, or in splenocytes. CRAM mRNA was also not detected in resting monocytic cell lines, but was strongly expressed in primary monocytes and THP-1 cells
15 upon activation with IFN γ and LPS. Both CRAM-A and CRAM-B mRNAs were induced, as detected by RT-PCR using exon 1- and exon 2-specific primers. In contrast to several other monocyte activation-induced genes, such as monokines (TNF α , IL-1, IL-6) and some cell-surface
20 antigens (Ho and Moore. (1994) Therapeutic Immunology 1:173). CRAM mRNA expression was not detectably inhibited by IL-10. Thus, CRAM expression in monocytes may be regulated via a different mechanism compared to that of several other activation-induced genes.

25 While most CKR genes lack introns, the genes for human CCR2 and mouse CXCR4 (*fusin*) both contain at least two exons and both have two alternatively spliced forms. CCR2A and CCR2B differ in the C-terminus (Charo, et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:2752), whereas
30 CXCR4 has two forms that differ by two amino acids at the N-terminus (Heesen, et al. (1997) J. Immunol. 158:3561). The two forms of CCR2 have identical ligand specificities, but differ with respect to which G α subunits they can couple (Kuang, et al. (1996) J. Biol.
35 Chem. 271:3975); the two forms of CXCR4 can both serve as functional CKRs for SDF-1 α (Heesen, et al. (1997) J. Immunol. 158:3561), although their ligand specificities and interactions with HIV have not been fully characterized. The N-terminal sequence of CKRs, along

with portions of the extracellular loops, is known to play a key role in ligand binding and possibly receptor activation (Ahuja, et al. (1996) J. Biol. Chem. 271:225; Lu, et al. (1995) J. Biol. Chem. 270:26239; Horuk (1994) Immunol. Today 15:169; Wells, et al. (1996) J Leukoc Biol 59:53; and Hebert, et al. (1993) J. Biol. Chem. 268:18549). This region of CKR is also important for HIV fusion (Rucker, et al. (1996) Cell 87:437.), which is antagonized by chemokine ligands (Paxton, et al. (1996) Nature Med. 2:412; and Cocchi, et al. (1995) Science 270:1811). Thus it is possible that CRAM-A and CRAM-B may exhibit different but likely overlapping ligand specificities. Regulated expression of alternative forms of a single CKR, combined with possible modulation of specificity of ligand-receptor interaction by chemokine-proteoglycan interaction (Graham, et al. (1996) The EMBO J. 15:6506; and Witt and Lander (1994) Curr. Biol. 4:394), might control the spectrum of chemokines to which a particular cell could respond. In addition, these observations may provide one possible explanation of non-reciprocal desensitization phenomena observed with, for example, the chemokines RANTES, MIP-1 α , and MCAF (Wang, et al. (1993) J Exp Med 177:699).

The similarity of CRAM to the other β -CKRs, its chromosomal localization in the β -CKR gene cluster, and induction of its expression in monocytes upon activation all argue that CRAM may play an important role in regulation of immune function.

30

VIII. Screening for receptor/ligand

Labeled reagent is useful for screening of an expression library made from a cell line which expresses a chemokine or receptor, as appropriate. Standard staining techniques are used to detect or sort intracellular or surface expressed ligand, or surface expressing transformed cells are screened by panning. Screening of intracellular expression is performed by

35

various staining or immunofluorescence procedures. See also, e.g., McMahan, et al. (1991) EMBO J. 10:2821-2832.

For example, on day 0, precoat 2-chamber permanox slides with 1 ml per chamber of fibronectin, 10 ng/ml in
5 PBS, for 30 min at room temperature. Rinse once with PBS. Then plate COS cells at $2-3 \times 10^5$ cells per chamber in 1.5 ml of growth media. Incubate overnight at 37° C.

On day 1 for each sample, prepare 0.5 ml of a
10 solution of 66 µg/ml DEAE-dextran, 66 µM chloroquine, and 4 µg DNA in serum free DME. For each set, a positive control is prepared, e.g., of huIL-10-FLAG cDNA at 1 and 1/200 dilution, and a negative mock. Rinse cells with serum free DME. Add the DNA solution and
15 incubate 5 hr at 37° C. Remove the medium and add 0.5 ml 10% DMSO in DME for 2.5 min. Remove and wash once with DME. Add 1.5 ml growth medium and incubate overnight.

On day 2, change the medium. On days 3 or 4, the
20 cells are fixed and stained. Rinse the cells twice with Hank's Buffered Saline Solution (HBSS) and fix in 4% paraformaldehyde (PFA)/glucose for 5 min. Wash 3X with HBSS. The slides may be stored at -80° C after all liquid is removed. For each chamber, 0.5 ml incubations
25 are performed as follows. Add HBSS/saponin(0.1%) with 32 µl/ml of 1M NaN₃ for 20 min. Cells are then washed with HBSS/saponin 1X. Add antibody complex to cells and incubate for 30 min. Wash cells twice with HBSS/saponin. Add second antibody, e.g., Vector anti-
30 mouse antibody, at 1/200 dilution, and incubate for 30 min. Prepare ELISA solution, e.g., Vector Elite ABC horseradish peroxidase solution, and preincubate for 30 min. Use, e.g., 1 drop of solution A (avidin) and 1 drop solution B (biotin) per 2.5 ml HBSS/saponin. Wash
35 cells twice with HBSS/saponin. Add ABC HRP solution and incubate for 30 min. Wash cells twice with HBSS, second wash for 2 min, which closes cells. Then add Vector diaminobenzoic acid (DAB) for 5 to 10 min. Use 2 drops of buffer plus 4 drops DAB plus 2 drops of H₂O₂ per 5 ml

of glass distilled water. Carefully remove chamber and rinse slide in water. Air dry for a few minutes, then add 1 drop of Crystal Mount and a cover slip. Bake for 5 min at 85-90° C.

5 Alternatively, the binding compositions are used to affinity purify or sort out cells expressing the ligand or receptor. See, e.g., Sambrook, et al. or Ausubel et al.

10 All references cited herein are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

15 Many modification an variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of the equivalents to which such claims are
20 entitled.